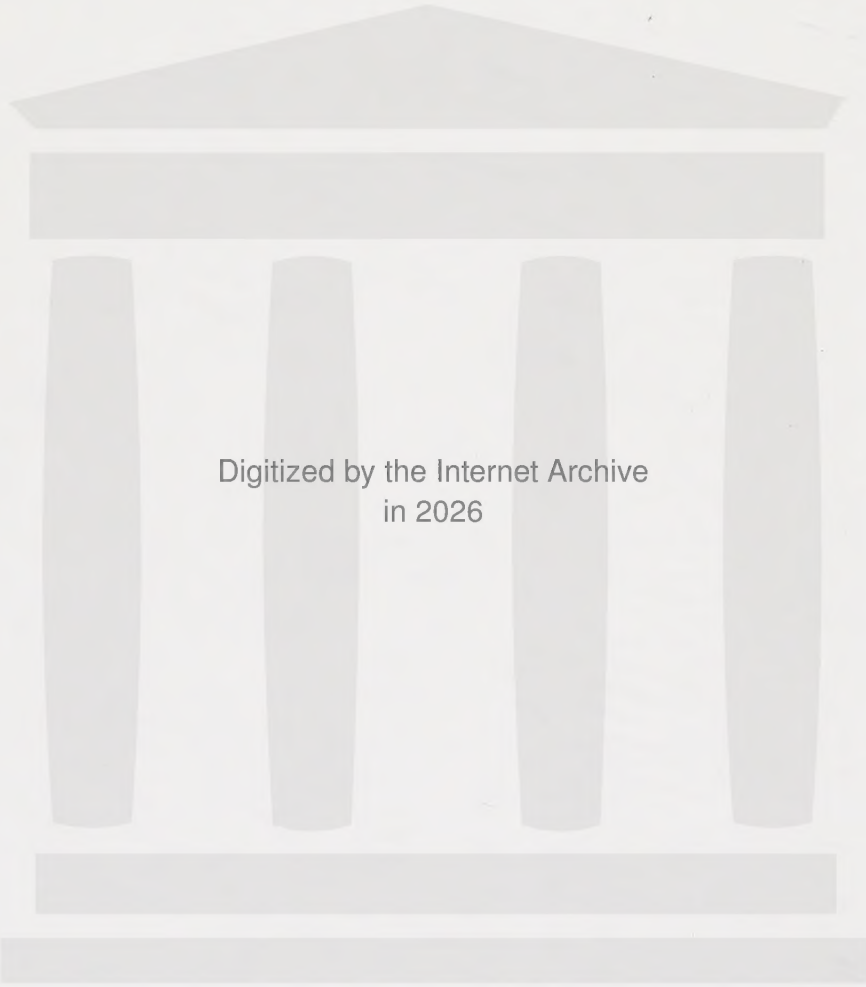


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STUDIES ON CELL DIVISION IN THE HUMAN EPIDERMIS

II. A. RATE OF CELL DIVISION IN THE PREPUCE

B. INFLUENCE OF VARIOUS FACTORS ON CELL DIVISION

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INTRODUCTION

The present status of our knowledge of stratified squamous epithelium has recently been summarized in several excellent reviews, notably by Patzelt ('26) and more recently by Pinkus ('27), Hoepke ('27), and Schaffer ('27). While the first three authors devote most of their attention to the stratified squamous epithelium of the human epidermis, the latter work deals with stratified squamous epithelium in general. Although the various phases of regeneration are discussed in great detail and from almost every conceivable angle, yet a compilation of the different findings results in an apparently irreconcilable mass of information.

This confusion is due to three things: 1) the difference of opinion as to whether amitosis is a factor in the proliferation of stratified squamous epithelium; 2) the disagreement as to the particular layers in which cell division occurs; and 3) the varying significance of the material selected for study.

The importance of considering amitosis a possible factor is constantly gaining ground; however, the conditions which give rise to this form of cell division are as yet not fully understood. The fact that in normal epidermis insufficient mitotic figures are encountered to compensate for the constant loss of cells from the surface, and the relative frequency with which binucleated cells may be observed, es-

pecially near the stratum granulosum, has strengthened the assumption that amitosis perhaps takes place along with mitosis.

As to the portion of the rete Malpighii actually concerned in cell division, Patzelt ('26), reporting on numerous fresh sections of human epidermis obtained from operations or executions, states that he observed mitosis chiefly in the basal cells and occasionally also in the second layer. Pinkus ('27) states quite emphatically that mitosis of the basal-cell layer constitutes the only normal mode of replacement of the epidermal cells. Flemming ('83), in studying the epidermis from various regions of different mammals, admits the difficulties encountered in finding mitoses until he observed them in great numbers in the snout of a full-grown pig. Here, they were confined to the deepest two or three layers of the rete Malpighii. Hoepke ('27), basing his opinion upon a thorough review of the literature, comes to the conclusion that the term stratum germinativum should be applied to the entire rete Malpighii, which includes both the single columnar-cell layer of the stratum basale and the variable number of cell layers belonging to the stratum spinosum. He is convinced that mitosis occurs more frequently than generally accepted. This is also in agreement with the conclusions of Thuringer ('24) and those of Schaffer ('27).

In a former study undertaken for the purpose of determining the actual site of cell proliferation in the human epidermis, serial sections of scalp obtained at executions were used (Thuringer, '24). The result of this work was greatly at variance with the previously accepted views expressed by most authorities, principally because in the epidermis of the scalp the majority of mitoses were encountered in the stratum spinosum. In fact, if the rete Malpighii were subdivided into four parts—an inner (the stratum basale), the lower, middle, and outer one-thirds of the stratum spinosum—the percentage of mitoses encountered was 12 per cent, 30 per cent, 46 per cent, and 12 per cent, respectively. Thus, the principal site of proliferation was the middle one-third of the stratum spinosum.

Relative to the varying significance of the material selected for studies of the human epidermis, the literature shows that a majority of observations were made on sections obtained from the palmar and plantar surfaces of the hands and feet which represent only one-twentieth to one-twenty-second of the general body surface. Investigations in comparative histology likewise reveal the fact that such efforts were mostly confined to highly specialized and restricted surfaces rather than to the general body surface.

The various regions of the epidermis, while showing the common function of surface protection, unquestionably are at the same time more or less highly specialized to meet the peculiar needs of a given region. The periodicity of cellular regeneration depends upon the functional demands of the epidermis. Since the function of this tissue is primarily protection of the surface, with consequently more or less constant loss of superficial cells, there must be present at all times compensatory regeneration.

With the thought of clearing up or perhaps reconciling some of this apparently conflicting information, the study of epidermis from other body regions was undertaken in order to determine, if possible, what factor or factors were responsible for the divergent views reported in the literature.

MATERIAL AND METHODS

The material selected for study was the normal prepuce of younger and older children. To avoid any detrimental effects which might be attributed to the usual aseptic preparations of the operative field, the prepuce was only sponged and rinsed with sterile water, the entire preparation being of less than ten seconds' duration. In order also to study the effect of fixation time upon the number of mitoses present, it was decided to fix parts of the skin at varying intervals after removal. The prepuce was clamped and removed in toto, a portion of it was immediately placed in Zenker's fluid, and from the remainder, pieces were cut off and fixed at intervals of five minutes. The tissues were embedded by

the combined celloidin-paraffin method and the serial sections of 10μ thickness stained with Mann's acid-haematein and eosin. The sections finally selected were from an infant of seventeen days and a boy three years old.

A careful search was made for mitotic figures in each of twenty-five serial sections. The results were tabulated according to their location in the stratum germinativum, their phase of mitoses, and, in case of the cells in the stratum cylindricum, also the angle of the axes of the dividing cells to the basement membrane.

A cell count was then made to determine the ratio of mitosis to total cells. Since the diameter of the nuclei was practically the same as the thickness of the sections, no particular difficulty was experienced in counting cells. A $\times 4$ eyepiece, provided with two slightly curved, parallel-arranged hairs, dividing the field into a narrow median sector and two lateral fields, was of great aid in enumerating cells around papillae. A hand-tally counter was found to be an indispensable piece of equipment.

OBSERVATIONS

In the determination of the rate of mitosis in a given section several factors should receive consideration: the age of the individual, the length of time elapsed from removal of the tissue until fixation, the region from which the tissue is taken, and the state of activity prior to and at the time of removal and fixation.

The age factor

The importance of the age factor is too well known to merit lengthy discussion; suffice it to say, the younger the individual and the more rapid the growth, the more mitotic figures one may expect to find. This is clearly illustrated in table 1 by comparing the ratio of mitoses in the instantly fixed prepuce of seventeen days and that of three years. In the former it was found to be 1:178.2, while in the latter it has decreased to 1:1359.

The fixation factor

The importance of the time of fixation as a factor influencing the presence of mitotic figures has at various times been emphasized and at other times considered unimportant. Patzelt ('26) states that "the short duration of mitosis . . . does not afford a basis for the relatively infrequent encounter of mitotic figures in the human epidermis." He examined about 400 sections of instantly fixed fresh human material, and reports that he found numerous mitoses only in one section from the sole of the foot, and that very one was not fixed until twelve hours after death. This, however, in our mind constitutes no proof that an even greater number might

TABLE 1
Rate of mitosis in epidermis from various parts of the body

SOURCE OF TISSUES	TIME OF FIXATION	NUMBER OF SECTIONS COUNTED	TOTAL CELLS COUNTED	TOTAL MITOSES	RATIO
17-day prepuce	Instantaneous	25	81,264	456	1:178.2
3-year prepuce.	Instantaneous	25	188,200	138	1:1359
Adult scalp	Instantaneous	50	410,440	170	1:2414
Adult ear	Instantaneous	50	1,609,650	6	1:268,275
Adult leg	Instantaneous	25	378,325	1	1:378,325
Average for various portions of epidermis		175	2,667,879	771	1:3460

not have been observed had the material been fixed instantly. Evans ('26), studying the effect of fixation time upon cancer tissue, one a mixed-cell sarcoma of the thigh, the other a spindle-cell sarcoma, came to the conclusion that there is no material variation in the number of mitotic figures present in sections, whether the tissue be fixed instantly or if it be kept for twenty-four hours at room temperature, ice-box, or incubator temperature. He states, "there is, however, in each case evidently a slight drop in the number present." It is to be noted that his counts do not represent absolute values for a series of sections, but were made by enumerating all the mitotic figures in approximately one hundred oil-immersion fields.

The influence of fixation time becomes evident by consulting table 2, where it is shown that the ratio of resting cells to mitoses increases with the delay in fixation. Had we been able to carry these experiments on through longer time intervals we should more than likely have found a period beginning with which no additional change in the ratio of mitosis would be encountered. Practically all mitoses observed in the instantly fixed material were normal. In the five-minute material a few figures were found which showed signs of degeneration. The latter forms increased in number so that in the ten-minute material about half of the mitotic figures

TABLE 2

Rate of mitosis in prepuce to show effect of age and time of fixation

SOURCE	TIME OF FIXATION	NUMBER OF SERIAL SECTIONS	TOTAL CELL COUNT	TOTAL MITOSIS	RATIO
17-day human prepuce	Instantaneous	25	81,264	456	1:178.2
	5 minutes	25	88,784	320	1:273.1
	10 minutes	25	102,540	245	1:418.5
	15 minutes	25	99,825	157	1:633.8
3-year human prepuce	Instantaneous	25	188,200	138	1:1359
	5 minutes	25	419,825	82	1:5120
	15 minutes	25	354,250	30	1:11,814

Note: The differences in the total cell counts are due to the inequality in size of the various pieces of tissue.

showed marked degenerative changes, while in the fifteen-minute material practically all cells gave evidence of degeneration in varying degrees.

Mitoses in the prophase stage were present in the greatest number. These were followed in number by the telophase, anaphase, and metaphase, respectively. The relatively small number of mitoses in metaphase and anaphase attest the rapidity with which the cells pass through the cycle. Lewis ('17) determined the duration of the entire mitotic cycle in tissue cultures of mesenchyme as being from 70 to 180 minutes. Patzelt ('26), quoting Peters and others, assumes the time for mitosis in Mammalia to be about half an hour.

The regional factor

The regional factor probably has not been given as much consideration as it merits. The parts of the epidermis that are constantly exposed to pressure, friction, or great thermal changes must necessarily regenerate at a more rapid rate than those portions of the skin not subject to such environmental factors. The volar and plantar surfaces of the hands and feet are unlike most other portions of the epidermis; we are only too familiar with their prompt responses to stimuli in the form of calluses; whether or not the mechanism of regeneration of this highly specialized surface is the same as that of the general body surface, is another question. The physiological activity of any given region must also be taken into consideration; one would not expect that regeneration of the plantar epidermis would be alike in an individual walking or one lying in bed.

Referring to table 1, we find that fifty sections from the ear, representing a surface area of 21.3 sq.mm., gave a total cell count of 1,609,650 with six mitotic figures; while twenty-five sections taken from the anterior tibial region, and having a surface area of 4 sq.mm. with 378,325 cells, showed only one mitotic figure. The ear and scalp were from one individual (execution), while the skin of the leg was from an amputation. Compared to the figures from the scalp with 10 mitoses per sq.mm., an equal area from the epidermis of the ear showed but 2.8 mitoses, while in a similar area from the leg 2.5 mitoses were found.

In examining sections from the prepuce, aggregations of mitotic figures were frequently found extending through an average of about ten serial sections of 10μ thickness, the diameter of the largest field constituting these 'growth-waves' being approximately 100μ . It was possible to count as many as ten to fourteen figures under high power ($\times 400$) in a single field through the center of a 'growth-wave,' while toward the periphery of these proliferation centers the number of figures would taper off to zero. This fact should be taken into consideration when attempting cell counts and it

also explains why cell counts made by selecting a number of fields at random may not give the desired information. To emphasize this point we desire to quote a paragraph from the work of Flemming ('84) in which he recorded his observations on local variations, calling attention to the occurrence of cells growing 'schubweise,' i.e., in shifts: "It is striking, and of peculiar interest for the explanation of the many failures, that mitoses occur almost everywhere in local groups. Where one is found, others are encountered in adjacent areas, and on the other hand, great areas, occasionally entire sections of more than 1 cm. length may be entirely without mitoses. Had I, casually, at the beginning of my investigation, encountered such an unfavorable section, I would in all probability have laid aside the entire object with the report of not having seen any mitoses. This may have been the cause of many a fruitless search in my previous work as well as in that of others." The following year, while studying the epidermis of adult rabbits, guinea-pigs, and cats, Flemming ('85) was able to confirm his suspicions relative to the appearance of mitotic figures in groups or localized areas while none could be found in adjacent portions. Our studies on the human epidermis are in complete agreement with the observations of Flemming.

Table 3 presents an analysis of the mitoses on the basis of their location in the rete Malpighii. The material chosen for this table is that of the seventeen-day prepucce, because it permitted the comparison of four successive specimens in which the fixation time was the only variable factor. We have again divided the rete Malpighii or stratum germinativum into four parts beginning with: 1) the columnar cells constituting the basal layer or stratum cylindricum; 2) the lower, 3) middle, and 4) outer thirds of the stratum spinosum. It is of interest to note the percentage of cells in the stratum basale and to compare the figures with the totals obtained from the three arbitrary divisions of the stratum spinosum. In the instantly fixed material this was 8.33 per cent for the former and 91.67 per cent for the latter. The percentage

for the basal-cell layer then rises very rapidly during the five-minute interval, reaching 12.89 per cent against 87.11 per cent in the stratum spinosum. During the next two intervals of five minutes the number of mitoses in the basal-cell layer is again on the decline, while the lower one-third of the stratum spinosum (fixed at fifteen minutes) shows an increase of about 5 per cent in the number of mitoses compared with the instantly fixed material.

TABLE 3

Analysis of mitoses according to location in the stratum germinativum (in four specimens from the prepuce of a seventeen-day boy)

TIME OF FIXATION	NUMBER OF MITOSES	STRATUM CYLINDRI- CUM	STRATUM SPINOSUM			TOTAL
			L-1/3	M-1/3	U-1/3	
Instantaneous	Total mitoses	38	347	70	1	456
	Total per cent	8.33	76.1	15.35	.22	100
After 5 minutes	Total mitoses	41	228	46	3	318
	Total per cent	12.89	71.7	14.47	.94	100
After 10 minutes	Total mitoses	29	189	26	1	245
	Total per cent	11.8	77.1	10.6	.5	100
After 15 minutes	Total mitoses	16	128	11	2	157
	Total per cent	10.2	81.5	7	1.3	100
Summary of four specimens	Total	124	892	153	7	1176
	Average number	31	223	38.25	1.75	294
	Average per cent	10.6	76.6	13.0	.6	100

In the stratum basale we failed to observe dividing cells which were about to be pushed into the stratum spinosum to become a part of that layer. The axes of the spindles were always parallel to the basement membrane and no evidence of telokinesis, as described below, was seen, thus giving one the impression that the new cells contributed either as replacements, or served to increase the surface area of this layer. The figures given in table 3, together with these observations, tend to substantiate our former contention that the stratum basale of the adult stratified squamous epithe-

lium serves to maintain the integrity of the epithelial tissue rather than to furnish new cells to the superimposed stratum spinosum.

The direction of the axes of the dividing cells in the stratum spinosum was always somewhat oblique to the vertical plane, a few exceptions were nearly horizontal. With the beginning of the telophase the spindles almost invariably began to bend upon themselves (telokinesis), the degree of bending varying with the extent of the cleavage of the cytoplasm. Often two daughter cells in late telophase were seen with their spindles bent at right angles and others, that remained connected only by a delicate stalk, showed that the axes of the spindle had bent until they formed a 'V.' This was interpreted as evidence of two-dimensional growth, namely, outward and laterally.

Comparing these figures with the data which were previously obtained on mitosis in the scalp, the average percentage of mitoses in the basal-cell layer of 10.6 per cent agrees very closely with the figures given for the scalp, 12 per cent. In the stratum spinosum of the prepuce 89.27 per cent mitoses were found compared to 88 per cent in the same layer of the scalp. The distribution of the mitoses within the stratum spinosum in the prepuce, however, was different from that of the scalp and more in harmony with the observations of Flemming. Whereas only 11.8 per cent of the mitoses were present in the middle one-third of the stratum spinosum in the prepuce, an average of 46 per cent were found in the scalp. The lower one-third of the stratum spinosum contained the greatest number of mitotic figures, an average of 76.6 per cent compared to the same area from the scalp with 30 per cent. The outer third of the stratum spinosum contained an average of only 0.6 per cent as compared to the 12 per cent found in the scalp.

The uniformity of the percentages obtained in the stratum basale of the prepuce and scalp is considered of greater importance than the difference encountered in the percentages of the various parts of the stratum spinosum. The fact

remains, nevertheless, that the total figures for the stratum spinosum are surprisingly in agreement for the two widely separated regions, namely, 88 per cent for the scalp and 89.27 per cent for the prepuce.

CONCLUSIONS

1. The process of regeneration of the epidermis is dependent upon the physiological requirements of the particular region from which the tissue is taken. Regeneration progresses faster, normally, in some regions than in others and is also contingent upon the state of activity of the region from which it is obtained. It is uniformly greater in the young than in those advanced in years.

2. The majority of mitotic figures encountered in the prepuce were in the stratum spinosum, the totals for the entire stratum spinosum agreeing within about 1 per cent of the figures given for the scalp.

3. The greatest proliferation centers, however, in the prepuce were found in the lower one-third of the stratum spinosum (76.65 per cent), in which respect it differs from the same layer in the scalp (30 per cent).

4. This study emphasizes the individuality of both the basal-cell layer and of the spinous-cell layers in the epidermis of the general body surface. Only 10.75 per cent of all mitotic figures were found in the basal layer, the axes of mitoses being invariably parallel to the basement membrane. Telokinesis was absent.

5. The importance of the fixation time is stressed. The number of mitotic figures observed was proportional to the time of fixation. The latter, therefore, is of paramount importance in the accurate estimation of the rate of regeneration.

6. Mitosis in the prepuce was localized into definite areas. These 'growth-waves' reached a maximum diameter of about 100 μ , containing in the principal area as high as fourteen mitotic figures in a single high-power field.

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THE PHAGOCYtic CELLS (v. KUPFFER) IN THE LIVER OF COMMON LABORATORY ANIMALS¹

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THREE HELIOTYPE PLATES (SIXTEEN FIGURES)

Since it has been shown that the reticulo-endothelial system, with its manifold subdivisions, is intimately related to both physiologic and pathologic processes, it becomes increasingly important that complete data should be compiled concerning the origin, function, and ultimate disposition of the cells comprising it. Our immediate interest in the reticulo-endothelial system is essentially restricted to the liver, in which the stellate cells, originally described by v. Kupffer, comprise a structure whose function is at once defensive and metabolic.

Relatively little is known concerning the interrelationship of stellate cells with the physiology and pathology of the liver. Schilling ('09) and Jaffé ('27) showed clearly that these cells have a definite relation to fat metabolism. Jaffé² showed that the stellate cells may engulf large quantities of lipid from the blood stream and subsequently transfer it to the adjacent hepatic cell. McNee ('22) credited the stellate cells with still another function, the formation of bile pigment, but there may be some question regarding this function, particularly in mammals. The relation of the stellate cells to iron metabolism is well established and an iron-staining technic will in-

¹ Submitted for publication June 20, 1928.

² Personal communication.

variably reveal it in the cells. In hemosiderosis, iron is always abundant in these cells and is transferred directly from them to the hepatic cells. Dick ('25) showed in cases of hemolytic jaundice that v. Kupffer cells of the liver are the chief agents of phagocytosis for the blood stream. In pathologic states in which either the liver or the blood is involved, these cells become affected. Nathan ('08) recognized the transformation of the stellate cells into fibroblasts in cirrhosis of the liver, and Maximow ('25) showed that in hepatic inflammation they may increase rapidly in number, become detached from the hepatic endothelium, and move as independent histiocytes in the blood stream. The fact that these stellate cells may increase rapidly in the absence of other portions of the reticulo-endothelial system, as Krumbhaar and Musser ('23) have shown following splenectomy, clearly suggests their vital relations to general metabolic activity.

The liver is known to have a certain defense mechanism, as shown by Mann and Bollman ('26), for the dehepatized animal is not able to withstand the effect of intravenous injection of toxic substances to the extent of an intact animal. Whether this detoxifying effect may be ascribed to these star-shaped cells is questionable. It is, nevertheless, certain that one essential function of them is the elimination of bacteria or foreign particulate matter from the blood stream. Ponfick ('69) showed, however, that when he injected cinnabar into the peripheral circulation of an animal, it finally reached certain cells of the liver, which were not of the hepatic parenchyma. The most extensive observations were made by v. Kupffer in 1876. Subsequently, more detailed studies were carried on and a complete report of his results was published in 1899. He demonstrated that the wall of a hepatic sinusoid is a syncytium and that the scattered 'star' cells project slightly into the blood stream and readily phagocytose. His observations have been entirely confirmed by later investigators.

Schilling ('09) identified three types of cells within the endothelium which lines the intralobular capillary wall. Only certain of these, which he described as more narrow in form

and more marked in staining reactions, are the true stellate cells. The other cells are endothelial in a more restricted sense, but they may become functional stellate cells under essentially stimulating physiologic conditions. Likewise, Zimmermann ('23) recognized three types of cells which comprise the wall of a sinusoid. Of these, only the inner cells which project from the endothelium into the blood stream and were designated by him 'endocytes,' are functional v. Kupffer cells. Havet ('25) in studies of embryonic vertebrates as well as certain adult vertebrates, concluded that the v. Kupffer cells of the liver are not endothelial cells, but that they arise in conjunction with those cells of endodermal origin which give rise to the liver. These cells lie beneath the capillary endothelium, between it and the hepatic trabeculae, and Havet regarded them in their relation to the hepatic cell as analogous to the neuroglia surrounding nerve cells. Such an interpretation of the position of the v. Kupffer cells is exceedingly difficult to correlate with the known facts concerning the protective function these cells discharge. The most recent study of the v. Kupffer cells has been made by Pfuhl ('26). On the basis of their form, position, and arrangement, Pfuhl identified three types of cells. He differentiated the 'seizing' type of cell from the 'digesting' type. The former projects more into the lumen of the sinusoids and is the stellate cell with phagocytic functions; the digesting cell is more ovoid, less exposed, and lies in small crypts between adjacent hepatic cells.

Our study of the stellate cells was approached with definite objectives. Although many descriptions of these cells are available, there is no adequate comparative study of them as they occur within the group of vertebrate animals. Nathan studied the comparative anatomy of v. Kupffer cells in a number of vertebrates, but only slight details were reported and his figures are not extensive. The origin of these cells from primitive vascular endothelium is relatively unknown, as is the extent to which these cells may phagocytose in fetal life. What is the life cycle of v. Kupffer cells and what is

their fate after ingestion of particulate matter? In certain pathologic states these cells become active polyblasts within the blood stream. Is this true in the normal animal? These are illustrative of the questions that need to be answered before the cytologic physiology of the liver is understood. This report is concerned only with the first of these inquiries and is restricted to mammals. Studies are under way on the livers of lower vertebrates also.

METHOD OF STUDY

The use of vital dyes in the delineation of the stellate cells has been for the most part satisfactory. India ink in dilute suspension has been frequently used as well as the iron stains. Since Drinker and Churchill ('27) described the graphite known as 'Hydrokollag 300,' we have found it a most excellent medium of injection for the study of the stellate cells of the liver. Our method of preparing the material for injections differs somewhat from that employed by Drinker and Churchill and is more rapid. In brief, it is as follows:

To 120 grams of the crude product, received from the manufacturer, 2 cc. of a saturated solution of sodium hydroxide in 250 cc. of distilled water is added. The mixture is then placed high over a Bunsen flame and care is taken that the temperature in the flask does not rise above 40°C. At the same time, a current of air is passed rapidly through the suspension to assist in driving off the free ammonia. Approximately an hour elapses before the odor of ammonia vapor disappears.

The solution of acacia and the sodium chloride, as noted by Drinker and Churchill, is then added to the suspension, and the entire mixture is centrifuged for ten minutes at moderate speed. Any flocculent material that may collect on the surface, adherent to the tube of the centrifuge, is removed with a spatula and the upper four-fifths of the suspension is pipetted off into a clean flask. The remainder of the fluid is decanted into a second clean flask and the residual cake at the bottom of the tube is discarded. The process of centrifuging is then repeated and, as would be expected, the amount of

flocculent material at the surface and the residual cake at the bottom of the tube is appreciably less following each successive period of centrifuging. It is always safer to be a trifle wasteful in pipetting off the suspension, rather than to assume the risk of preparing a solution that might possibly contain particles sufficiently large to result in embolism following intravenous injection. It is desirable to make a microscopic examination of the suspension before injection to determine the actual size of the particles and thus to guarantee an even, homogeneous suspension of the particulate matter. The final suspension employed in the injection is beautifully homogeneous, composed of particles no larger than erythrocytes and frequently much smaller. When every precaution has been followed in the preparation, we have never lost an animal from the intravenous injection of small quantities. It is evident that the slight changes from the procedure originally described by Drinker and Churchill are directed toward the saving of both time and effort and are only lacking in the intricacies of a much more detailed method of preparation. Furthermore, we have discontinued the use of blood sera and find that our results are equally satisfactory. Intravenous injections during these experiments have been made both peripherally into the vein of the leg and centrally into the mesenteric vein, and in either case the animals ordinarily did not sustain reactions.

The usual method employed throughout this study was to inject small quantities of 'Hydrokollag 300'—the amount depending on the size of the animal—directly into the venous circulation.

At varying intervals following injection, the animals were killed and the livers fixed in corrosive acetic and stained with hematoxylin and eosin. In a few cases the liver was perfused with physiologic sodium-chloride solution before the animal was killed, but since the stellate cells might be dislodged thereby, the method was discontinued. Complete fixation may be obtained by immersing small portions of the liver in the fixative for a period of from fifteen to twenty minutes, but

more satisfactory results followed fixation *in situ* by the introduction of the corrosive acetic directly into the portal vein.

Our study of the distribution of these stellate cells has been made on the dog, cat, swine, rat, rabbit, guinea-pig, pocket gopher, striped gopher, and monkey.

OBSERVATIONS

If a few cubic centimeters of this graphite preparation is injected intravenously into a dog, the blood stream will be relatively free of the particles within from ten to fifteen minutes. The reticulo-endothelial system throughout the body has phagocytosed the particles and the spleen, liver, and lymph nodes are relatively black with it. In studying the liver of these animals from forty-five minutes to an hour after the injection of the graphite, the material is found literally packed into certain of the cells lining the intralobular capillaries. Occasionally, a few particles may be observed loose in the blood stream and rarely, a polymorphonuclear leucocyte containing graphite granules is seen. Then too, a number of circulating blood histiocytes, which contain varying quantities of the particulate matter, abound in the sinusoids together with the erythrocytes. These are the large mononuclear cells and in some instances they are large enough to completely fill an entire sinusoid.

By far the greater amount of the injected material is stored in the stellate cells of the liver. These cells are readily discernible and in the low-power field (fig. 1) they present a marked stippled contrast to the eosin-stained cells of the trabeculae of the liver. In the canine liver the hepatic lobules are not so completely delineated from each other by connective-tissue sheaths, as in certain other animals, and the radial arrangement of the sinusoids toward a central vein is not so pronounced. The hepatic trabeculae and thus the sinusoids are profusely branched and frequently the cross diameter of the latter is smaller than the cross-section area of a single hepatic cell. It is quite easy to recognize the endothelial lining of these sinusoids, although cell bodies are not always

apparent; occasionally, the delicate cytoplasmic processes of these cells have retracted somewhat from contact with the hepatic cell. These processes, which are definitely a part of the endothelium and considerably removed from the cell body of which they are a part, do not appear to phagocytose. But other cells of the endothelium which project more into the lumen of the sinusoid comprise those physiologically phagocytic cells known as the stellate cells of v. Kupffer. In some instances the cells are truly enormous and their stellate form is exceedingly conspicuous. In others the cells are distinctly oval in outline and the graphite is restricted to a zone immediately around the nucleus. Occasionally, stellate cells have been observed whose greatest length has exceeded the combined diameters of two or three hepatic cells. In such cases the prolonged processes course along the margin of the hepatic cells or, as is frequently seen, they may even extend directly across the sinusoid and follow the hepatic cell of the opposite side. Occasionally, more than one such strand may project directly across the sinusoid or frequently, free processes may terminate abruptly in the center of the lumen (fig. 2). Although there is some evidence to the contrary, the stellate cells that are the more closely packed have phagocytosed the more particulate matter and are situated at or near the confluence of two or more sinusoids. Thus, these cells would appear to be guarding a double portal and are more likely to discharge their function adequately.

Two or more types of cells undoubtedly comprise the endothelial lining of these intralobular capillaries. Numerous oval cells, resembling typical endothelial cells, lie closely applied to the hepatic parenchyma without particulate inclusions. These frequently lie in the interstices between projecting margins of the hepatic cells. The cells are invariably without graphite particles and the question arises whether they may be immature or perhaps held in reserve for future use. Occasionally, we have noted capillary walls comprised of two or three such cells. Of these the outer cells were well filled with graphite, but the inner were entirely devoid of it. One may

postulate a proliferative activity here, in the sense that the deeper layer of cells may give rise to the outer phagocytic areas. Although the regenerative capacity of the stellate cells has never been established, it is entirely probable that such restoration may take place. The frequency with which histiocytes are found in the blood in proximity to attached stellate cells suggests the transformation of the one into the other, as was observed by Maximow in certain lesions of the liver. These relationships in normal physiologic processes are as yet entirely unknown. Our observations support those of Zimmermann ('23) in that the phagocytic cells are the outer ones (those projecting into the blood stream, and called by him endocytes), but we are not able entirely to confirm the observations of Pfuhl ('26) that the different types of cells may be classified on the basis of their seizing or digesting activities.

The structure of the liver in the cat does not essentially differ from that of the dog. The lobules are not distinct and the radial distribution of the sinusoids and the hepatic trabeculae around the central vein are not as conspicuous as in swine. Intravenous injections of small quantities of the graphite are readily phagocytosed by the v. Kupffer cells of the liver, and in stained sections their contrast to the hepatic parenchyma is quite striking, although the distribution of these stellate cells in the liver of the cat is not as great as in that of the dog (fig. 3). Caution should be exercised, however, in drawing conclusions as to the incidence of such cells on histologic analysis only. Physiologic activity may have packed certain of them to such an extent that they may have become dislodged and carried away as blood histiocytes. These statements are, of course, postulates and perhaps unwarranted. Most of the stellate cells present resemble in many respects those of the dog. The cells are larger, however, and their nuclei usually spherical in outline and surrounded by densely packed cytoplasm, but attempt has not been made to secure details of the organization of the cytoplasm in these cells (fig. 4). The remaining cells of the capil-

lary wall which comprise the endothelium are more elongated and contain more ovoid nuclei than similar cells in the dog. Here, as in the dog, however, these typical endothelial cells do not phagocytose and, accordingly, all the cells lining the sinusoids are not phagocytic at any one time.

The liver of the swine is the one usually selected as the classical type for histologic study. Details of this organ are so familiar that we shall add only a little concerning its general arrangement or its division into component parts. It is sufficient to say that the liver is made up of a series of irregularly arranged hexagonal lobules from the peripheral angles of which strong bands of fibrous connective tissue pass outward, subdividing other areas of hepatic tissue into less regular masses, which may or may not contain an intralobular vein. In true transverse section, nevertheless, there is always the typical radial arrangement of sinusoids toward the central veins. Definite trabeculae are present and are composed of from two to three hepatic cells, frequently interrupted by a transverse anastomosing capillary branch. The hepatic cells correspond in size and structure to the usual mammalian type. However, the stellate cells appear to be less numerous than in any of the animals of this series (fig. 5) and, accordingly, the true endothelial cells are correspondingly increased in size and frequency. Deductions may be misleading, for one is never certain that the injection method has disclosed all functional stellate cells present in any lobule or group of lobules. Very few of these cells in swine appear to measure more than the diameter of any single hepatic cell, while most of them were found to extend only a fourth of this distance. The typically stellate forms of v. Kupffer cells were seen only occasionally. This may be explained, however, on the basis that an insufficient amount of the preparation had been injected, for we have noted that these cells have a definite relative proliferative power depending on the length of time and the amount of work they are expected to perform. However, so far as could be determined, few, if any, stellate cells are devoid of the graphite.

The liver of the rat (*Mus norvegicus albinus*) is not a typically well-defined mammalian liver, although it is made up of the usual characteristic lobules, which may be recognized macroscopically. It is much more deficient in its interlobular connective-tissue content than would be expected from a general survey of a microscopic section under low power. This rather deceiving 'unit liver organization' makes the detailed study of any such liver exceedingly difficult. The guinea-pig is probably the best illustration of this type of liver in any of the mammals we have studied. It is evident from microscopic examination just why the appearance, form, structure, and arrangement of these cells prompted v. Kupffer to apply to them the term, 'Sternzellen.' In any section cut through the liver of the rat one finds the orderly and regular arrangement in which the central veins make their appearance and the vivid radial pattern effect of v. Kupffer cells around these veins (fig. 6). The sinusoids, together with the stellate cells which make up part of their lining membrane, are, in a longitudinal section, perpendicular to these various intralobular veins. Usually, one may be sure to find these cells in any sinusoid into which a capillary opens at an acute angle (fig. 7). According to Pfuhl ('26), this is a strategic position and assists the cell to fulfill its physiologic function. The aggressiveness with which these cells are engulfed and packed with graphite material makes the detailed study of their finer structure relatively impossible. Likewise, observations on the normal cellular phagocytic content, including erythrocytes, leucocytes, pigment, and ordinary débris, are inhibited. The nucleus is seen occasionally and is usually single, although two nuclei are sometimes present. The stellate cells usually occupy about half of the sinusoid in which they appear and frequently they occlude the entire space, except at the point where the sinusoids branch. The average length of this special type of cell is equal to about a third of the length of any single hepatic cell, although many of them may extend the entire length of three hepatic cells.

A marked similarity exists between the liver of the white rat and that of the rabbit (fig. 8). In both animals the hepatic capillaries are arranged predominantly in a radial direction with transverse anastomoses occurring at a distance from one another of from four to six hepatic cells. In the liver of the rabbit, however, the evenly spaced central veins appear to be more frequent and regular, so that the lobules of the liver, as well as the cells of the liver, are smaller than those of the rat. Accordingly, these stellate cells appear relatively larger in comparison and on the average measure about half the dimension of a single hepatic cell, although here, again, they may extend three times this distance (fig. 9). The thickness of these cells is about equal to a third of their linear dimension. Because of the radial arrangement of the capillaries in the lobules of the liver in which they are separated from one another by only a single layer of cells, transverse or tangential sections between any two lobules appear as alternating strands of sinusoids and hepatic cells. The liver of the rabbit does not have true trabeculae, but those cords of the liver which are usually called trabeculae are simply rows of cells appearing between the meshes of the capillaries. These hepatic cells cannot form a meshwork, because the meshes of capillary network are much longer in the tangential than in the radial direction, as shown by Hering ('67). One may think of the capillaries, however, as a supporting structure, around which strands of hepatic cells are placed so that the so-called trabeculae are separated on all sides from their adjoining cells, and the stellate cells are accordingly actually increased in number. The distance between any two of them is frequently only one or two hepatic cells and often less. There is, however, a marked diminution in the number of endothelial cells forming the limiting membrane of the sinusoids. We agree with Schilling ('09) that the v. Kupffer cells form an integral part of the endothelial lining, appearing as little projections into the lumen of the capillary, and differing only in their physiologic function from the cells lining the capillary bed. Because of this, the ordinary endothe-

lial cells can be differentiated from these specialized stellate cells with ease. The former are smaller and apparently less numerous, ovoid in outline, and limited by a visible membrane. In our observations they have never been observed to phagocytose any of the injected material. On the other hand, the stellate cells, highly specialized endothelial cells, are considerably larger, irregular in outline, and in position directed toward the lumen of the capillary.

The liver of the guinea-pig, as has been stated, is exceedingly difficult to study because of the absence of any lobulation tending toward liver organization. Although frequent intralobular veins are present and irregularly spaced with associated fatty infiltration, the hepatic tissue gives the impression of a heterogeneous mass of hepatic cells. There is a tendency toward the radial outline, but since the hepatic cells are so closely arranged together it is difficult to identify any special pattern of the liver. Few, if any, transverse anastomosing capillaries are seen and, likewise, few sinusoids into which another capillary empties (fig. 10). The hepatic cells are irregular in shape and the cytoplasm is finely granular, containing an eccentrically placed, spherical nucleus. The stellate cells appear less numerous and smaller than those of certain other animals, and closely resemble those found in swine. They are relatively small, about two-thirds the dimension of a hepatic cell, and their width is about a third of their own length.

The liver in the pocket gopher (*Geomys bursarius*) resembles strikingly the structure of the liver of the white rat (fig. 11). The lobules of the liver are smaller, however, and more irregularly arranged, while the open-lace or meshwork pattern of alternating sinusoids and strands of hepatic cells is more evident. The capillaries are arranged radially and generally are compressed to include only a single layer of hepatic cells between them. Transverse anastomosing capillaries, ordinarily occurring at a distance of four to six hepatic cells, likewise exist in the liver of the gopher. Stellate cells appear with consistent regularity at the junction of a capil-

lary with an adjoining sinusoid. As in the rabbit, the liver of the gopher does not have true trabeculae. The stellate cells appear as voluminous, blackened, protoplasmic masses diffusely studding the entire field. They are decidedly numerous in this animal; many sinusoids, in any given section, may contain three, four, or sometimes five stellate cells (fig. 12). Numerous cells have been observed to extend five times the length of a single hepatic cell and of approximately the average width of one. On the average, these cells are equal to the dimension of ordinary hepatic cells and their width is approximately a fourth of their own length. They are of a distinctly blocky appearance and lack the finer protoplasmic extensions which have been seen in livers of other mammals of this series. They are, however, of greater dimension than any similar cells studied in this group. Stellate pseudopodia-like processes did not extend beyond the hepatic cells, as is often seen in the colloidal gold-impregnation method of staining of other workers.

The liver of the northern gopher (*Thomomys talpoides*) showed marked similarity to that of *Geomys bursarius*, described. In the structure of the liver it closely resembles the typical arrangement noted in all rodents (fig. 13). There are, however, a few distinguishing characteristics of the liver of the northern gopher that perhaps need further explanation.

The strands of the liver are usually three to four cells in thickness, alternating with sinusoids that are greatly diminished in size, so that the impression is given that the sinusoids are compressed on all sides by adjacent hepatic cells. The radial-like capillaries are definitely decreased in diameter and follow a straight course toward the central veins for a distance of four to five hepatic cells before transverse anastomosing capillaries appear. The hepatic cells of the northern gopher when compared to those of *Geomys bursarius* are about one and one-half times as large in either dimension.

In the liver of the striped gopher (*Spermophilus tridecemlineatus*) one notes the marked dissimilarity to the structure of the liver of the two pocket gophers described (fig. 14).

Central veins are present, but the radial pattern of capillaries is absent except for those lying in the immediate vicinity of the intralobular veins. There, they extend outward for a distance of from four to seven hepatic cells. Beyond these definitely circumscribed areas, the sinusoids appear as a multiplicity of minute, irregularly arranged spaces without any consistent design. Their size is so diminished that were it not for the decided contrast of the blackened stellate cells present, these areas would not be seen without difficulty. The hepatic cells are a trifle smaller than those present in the northern gopher, irregular in outline, and contain an ovoid, centrally placed nucleus.

Studies on these three types of livers reveal a striking similarity in the content of these stellate cells. They appear as voluminous, rather fine, elongated, protoplasmic masses scattered irregularly throughout the entire field. They are likewise numerous in these animals and any given section through a sinusoid is almost certain to contain a stellate cell which may completely fill its own sinusoid. Few, if any, branching sinusoids have been observed. The v. Kupffer cells of the striped gopher, on the average, measure the dimension of a single hepatic cell with a diameter of a fourth of its own length. Many cells, however, have been observed to extend the distance of four and sometimes five hepatic cells. The stellate cells in the striped gopher and the pocket gopher, although of entirely unrelated species, are about the same size. However, a comparison of these cells of the northern gopher with the size of its own hepatic cell, as was noted in the case of the pocket gopher, reveals a corresponding relative decrease in the proportions of this phagocytic cell in all of its dimensions. The appearance, distribution, and further cytologic study of the stellate cells of these two animals are so similar that further contrast is not warranted at this time.

The structure of the liver of the monkey resembles closely that of the dog, described, both in the absence of any definite lobulation, and in the scanty interlobular connective-tissue content (fig. 15). Nevertheless, lobules are present and re-

vealed through the arrangement of the blood vessels; yet, they do not show the independent contour observed in all the other animals except the guinea-pig. However, the lobules unite with one another around the periphery through their biliary-duct apparatus and their blood vascular system. Many hepatic cells are binucleate, but most of them contain only a single spherical central nucleus. The cytoplasm of the hepatic cell is finely granular and vacuole formation is occasionally seen. The injected material is found entirely within the stellate cells, which resemble closely those found in the dog both as to size, distribution, and approximate number; occasionally, they exceed in length those found in the dog. There are few capilliform sinusoids present that do not contain one or more stellate cells. They are usually a trifle longer than any single hepatic cell, and their shorter diameters measure approximately a third of this distance. Many of these cells with finely delicate protoplasmic processes have been seen to extend the distance of four and even five hepatic cells, coursing across a sinusoid and passing directly between two hepatic cells to an adjacent sinusoid (fig. 16). The nuclei of these cells are ovoid in shape and centrally placed; frequently, two nuclei may be present within a single cell, although mitotic figures have not been observed within them. Likewise, the normal intracellular phagocytic content has been found to include erythrocytes, leucocytes, and cellular débris. These stellate phagocytic cells are separated from one another by a distance of two or three hepatic cells, but we have never been able to recognize any organic continuity between them. Frequently, one or more endothelial cells have been seen lying between a single stellate cell and a hepatic cell.

COMMENT

These preliminary observations on the form, position, and frequency of the stellate cells of the liver in laboratory mammals lead us to conclude that these cells are actively phagocytic toward particulate graphite injected into the blood stream. Their response to the foreign substance is immediate; a frac-

tion of a cubic centimeter injected into a mesenteric vein is at once engulfed by these cells. They literally become engorged and, although we have taken sections of the liver immediately following intravenous injection, even then the stellate cells were well laden with the graphite, so much so that any detailed cytoplasmic study was impossible. Without the delineation of the cell, such as the injection method provides, an entirely inadequate picture of the phagocytic cells is gained. Their extent is never so completely realized as when it is seen after extensive phagocytosis of these particles of graphite, and one wonders whether such remarkable distensibility is not correlated in some way with the amount of work the cell must perform, and that in its resting, inactive state its form and size are considerably less conspicuous. The fate of these cells, following such remarkable distention as we have noted in some of our animals, is a question yet to be determined. It is logical to postulate the separation from the endothelium of the sinusoid and the continuation of such cells as blood histiocytes, previously shown to transpire in certain diseases of the liver. On the other hand, we have never recognized in the hepatic sinusoids any structure so definitely stellate or possessed of the long protoplasmic strands that would serve to identify dislodged v. Kupffer cells. Naturally, the section method has its limitations and one can never be certain, outside of methods involving tissue culture, whether such a transformation of endothelial cells into blood histiocytes occurs. Occasional polymorphonuclear leucocytes and macrophages are seen in the sinusoids, but our present data do not warrant any conclusion that identifies the latter cells with the stellate cells. If v. Kupffer cells are dislodged and continue in the circulation to the lung or elsewhere, one would expect the liver to become free of the graphite relatively soon. This is not the case, however, for the livers were not entirely free of the pigment three or four weeks following a single injection of the preparation. There is some evidence, as yet not entirely conclusive, that as the liver gives up the particles, the spleen becomes more heavily laden. These ob-

servations, however, need further confirmation, and a more detailed study must be made before the complete life-history of these cells is known.

These observations on the hepatic lobule of ten mammals have led us to conclude that the stellate cells are specialized cells contained within the hepatic endothelium. We are unable to substantiate the observations of Havet ('25) that these cells lie between the hepatic endothelium and the trabeculae of the liver; but we rather conclude with Schilling ('09), Zimmermann ('23), and others that these cells are an integral part of the endothelium, but directed in their position toward the lumen of the sinusoid. Such a position, with processes projecting into the blood capillaries, enables the phagocytic cell to function. The histogenetic relationship of these phagocytic cells to the remaining cells of the endothelium is not clear. The typical endothelial cell which limits the sinusoid and abuts against the hepatic cell is a much smaller cell, with elongated cytoplasmic processes and a single ovoid nucleus. Occasionally, two or more of these cells may lie superimposed on each other, and, as we have frequently noted, a single large phagocytic cell lies often on the lumen side of the other two. All three cells are apparently firmly adherent and together form what we believe to be the endothelium of the sinusoid at that site. Are these smaller, typically shaped endothelial cells progenitors of v. Kupffer cells or are they lineally independent of them? Conclusive evidence is lacking here with regard to this, but it does not violate probability to assume that one may transform into another. The ovoid endothelial cells are apparently not phagocytic. We have never observed the particulate matter within them; neither have we observed these foreign particles in the hepatic cells and we must conclude that the graphite does not pass as readily from the stellate to the hepatic cells as do certain lipoids during fat metabolism.

In all of the animals studied there was relative similarity in the size and proportions of the stellate cells. In certain animals the cells were more numerous, considerably larger,

and possessing a more definitely stellate form; in others they were more stocky and without the finer protoplasmic processes that are usually seen. Without regard to form or size, the exceedingly marked phagocytic activity of these cells was characteristic of all of the hepatic organs examined.

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PLATES

GEORGE M. HIGGINS AND GEORGE T. MURPHY

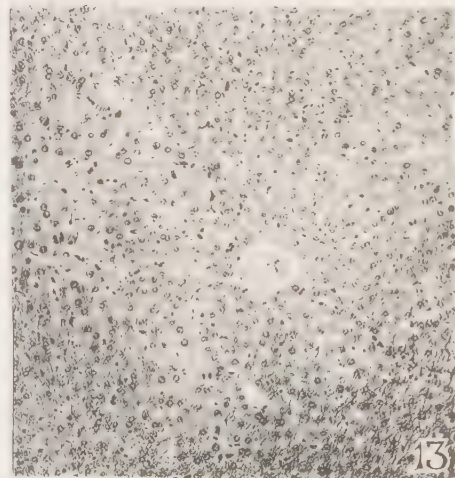
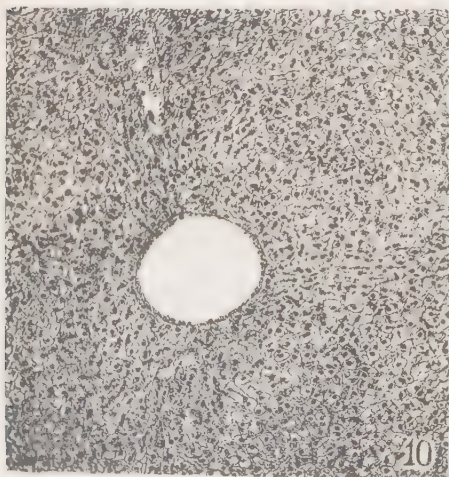
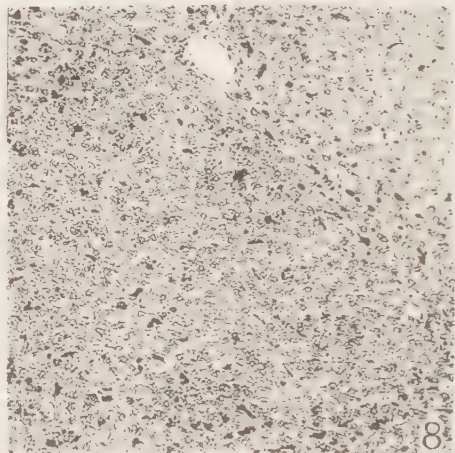
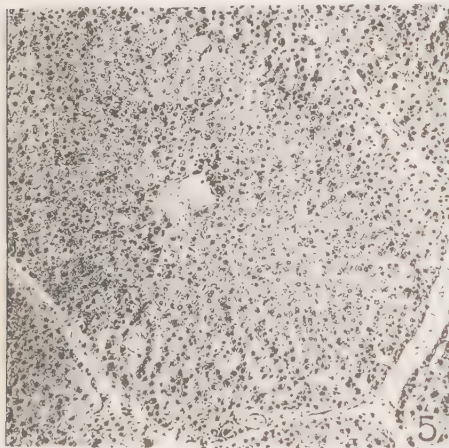
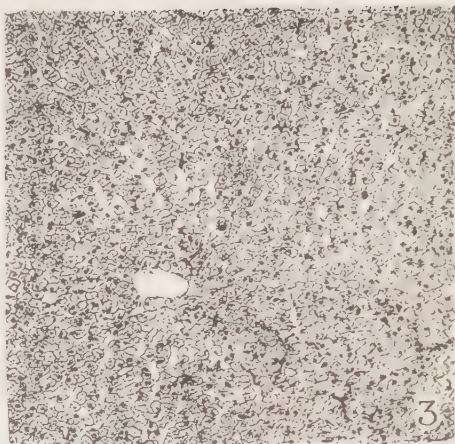
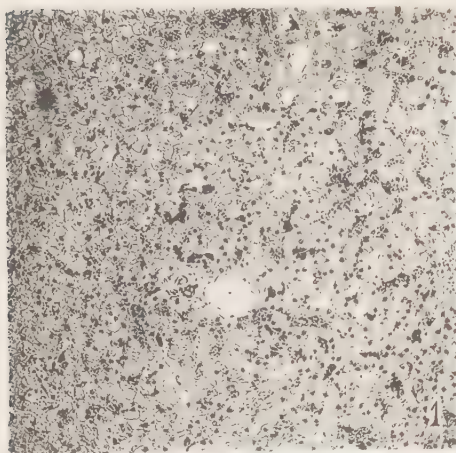


PLATE 1

EXPLANATION OF FIGURES

1 Section through the liver of a dog sixty minutes after the intravenous injection of 5 cc. of Hydrokollag 300. $\times 100$.

3 Section through the liver of a cat sixty minutes after the intravenous injection of 5 cc. of Hydrokollag 300. The black areas are stellate cells filled with graphite. $\times 100$.

5 Section through a lobule of the liver of a swine, showing characteristic form of lobule and the less numerous phagocytic cells. $\times 100$.

8 Section through the liver of a rabbit, showing the distribution of stellate cells. $\times 100$.

10 Section through the liver of a guinea-pig, showing the characteristic pattern. The sinusoids are reduced and are only recognized by the position of v. Kupffer stellate cells. $\times 100$.

13 Section through the liver of the northern gopher. A striking resemblance to the pocket gopher (fig. 12) is apparent in the arrangement of both sinusoids and stellate cells. $\times 100$.

•

PLATE 2

EXPLANATION OF FIGURES

6 Section through the liver of a white rat, showing the predominant radial arrangement of the sinusoids and the extensive development of v. Kupffer cells. $\times 100$.

11 Section through the liver of a pocket gopher. Sinusoids are well marked and stellate cells are accordingly well demonstrated. $\times 100$.

14 Section through the liver of a striped gopher, showing the reduced sinusoids, but nevertheless, well-marked stellate cells. $\times 100$.

15 Section through the liver of a monkey. The radial arrangement of sinusoids is not characteristic; but few are observed without actively phagocytic stellate cells. $\times 100$.

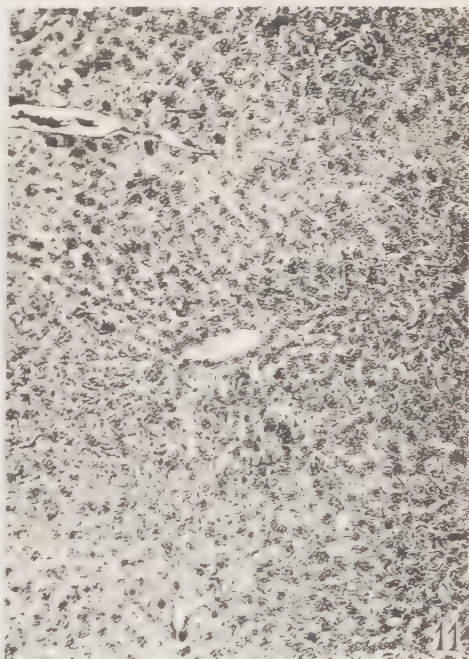
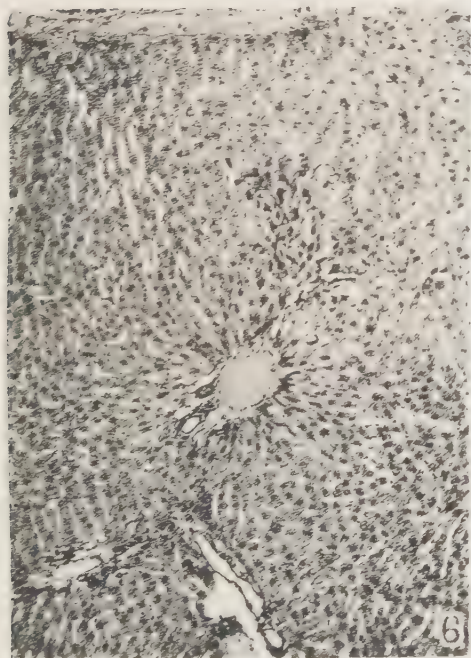
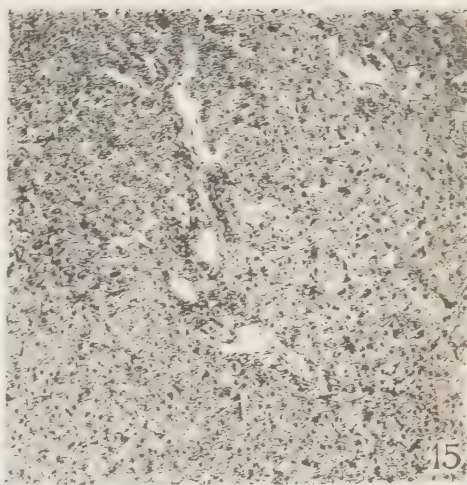
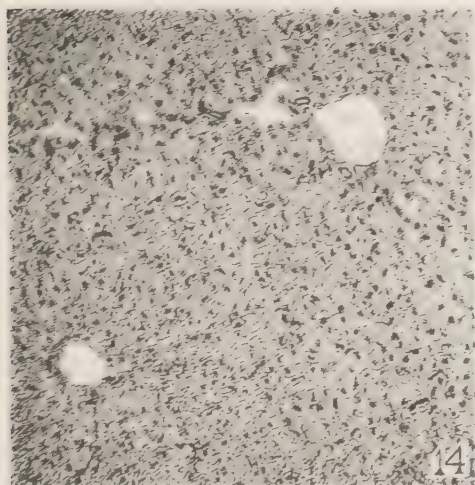


PLATE 3

EXPLANATION OF FIGURES

2 Section through the liver of the same dog as in figure 1, showing a characteristic grouping and size of v. Kupffer cells. $\times 900$.

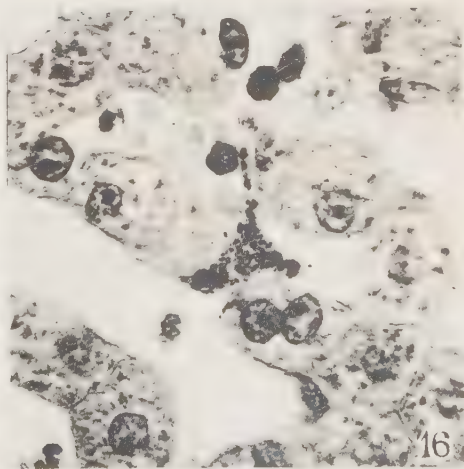
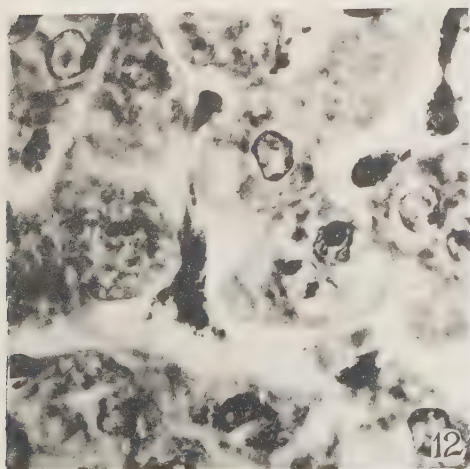
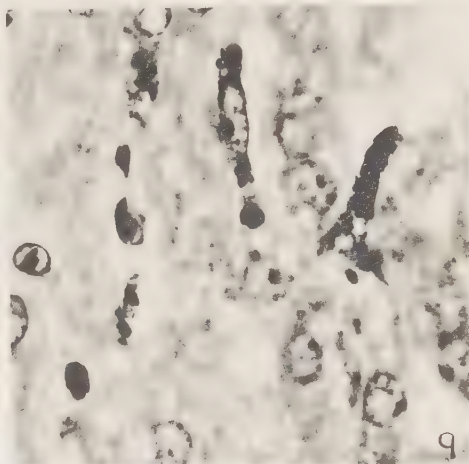
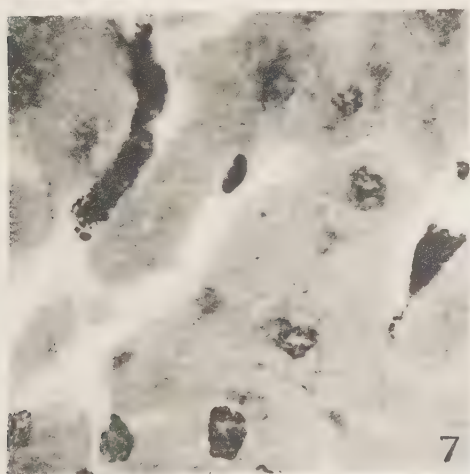
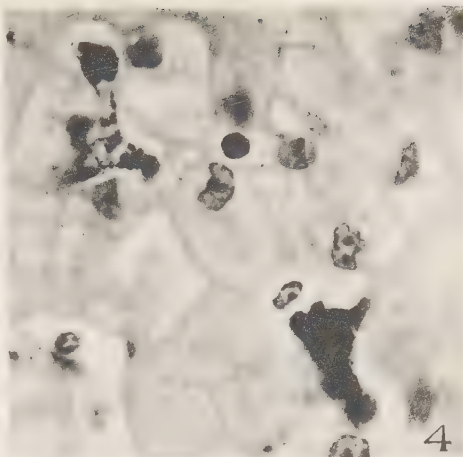
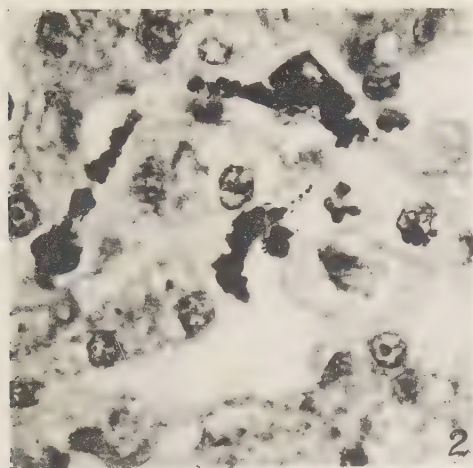
4 Section through the liver of the same cat as in figure 3, showing one of v. Kupffer cells completely filling a sinusoid. $\times 900$.

7 Section through the liver of the same rat as in figure 6, showing the extent of v. Kupffer cells as compared with hepatic cells. $\times 900$.

9 Section through the liver of the same rabbit as in figure 8, showing the characteristic elongated form of v. Kupffer cells in this animal. $\times 900$.

12 Section through the liver of the same gopher as in figure 11, showing one of v. Kupffer cells at the crest or saddle formed by the confluence of two sinusoids. $\times 900$.

16 Section through the liver of the same monkey as in figure 15, showing a typical stellate cell with extensive protoplasmic processes extending across adjacent sinusoids. Several polymorphonuclear leucocytes are seen containing portions of the colloid. $\times 900$.



A METHOD FOR THE MICROSCOPIC STUDY OF THE GROWTH OF TRANSPLANTED BONE IN THE TRANSPARENT CHAMBER OF THE RABBIT'S EAR

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SIX FIGURES

The method for the study of living cells and tissues in their normal environment in the transparent chamber of the rabbit's ear described by the author (Sandison, *Am. Jour. Anat.*, vol. 41, no. 3, 1928) has been shown to have opened a new field for the microscopic study with the highest magnifications of the growth and reaction of living cells and tissues in a mammal. A few studies on the growth and behavior of blood vessels under normal and experimental conditions have already been published and preliminary observations on endothelium, blood cells, fibrin, and macrophages have been carried out.

While the unmodified method has made possible the study of a vast number of problems, it was believed that its usefulness might be still further extended if tissues from other regions of the rabbit could be successfully transplanted to the chamber, where their character, growth, and behavior could be studied by daily observation in the living. This has been accomplished in the case of the transplantation of bone. Although the present paper contains merely a preliminary account of the growth and absorption of bone, an early publication of the work seems justified since the method makes possible the study of this tissue in a new manner, namely, by daily observations, in a mammal, of the same living cells with oil-immersion magnification. Moreover, it seems probable

that if bone can be successfully transplanted and studied in this way, a method has been developed for the study in the living of a large variety of transplanted tissues, under more favorable environmental conditions than those supplied by the tissue-culture method.

The technique of constructing the transparent chamber and inserting it into the rabbit's ear has been fully described (Sandison, '28) and will not be repeated here. The technique of the present method of obtaining bone growth in the chamber is exactly the same as that previously described, except that at the time of operation upon the ear the bone transplant is placed between the two pieces of celluloid. Two experiments have been performed: one, the transplantation of periosteum removed from the bone by scraping, and the other the transplantation of bone marrow which contained endosteum. In both experiments bone formed and grew beyond the region where the transplants were placed. Only the second experiment will be described in this paper since it offered a much better tissue for observation on account of the extreme thinness of the resulting growth.

The transplantation of the marrow and endosteum was done as follows: The chamber was constructed, filled with normal salt solution, and inserted into the rabbit's ear in the usual manner, with the main artery of the ear lying in the deep channel of the chamber provided for it. The sutures were left untied until later. Then the lower third of the tibia of the same animal (a young one) was amputated aseptically and a cylinder of marrow, about 8 mm. long and 2 mm. in diameter, was removed from the proximal end of the amputated portion of bone with as little disturbance to the marrow as possible. This cylinder of tissue, which contained presumably pieces of endosteum, was then placed inside the chamber and in contact with the main artery. The suturing of the chamber to the ear was then completed. As short a time as possible was used in making the transplant.

The region was observed from time to time, but the transplanted tissue was too thick and opaque to study in cellular

detail. Six weeks from the time of operation, however, it was discovered that new bone had developed which was extending into a very thin region of the chamber. Oil-immersion studies were immediately begun on the tissue of this narrow region and they have been carried on daily for one month. The tissue, which is richly supplied with blood vessels, is still alive and growing, and it remains to be seen whether it will survive as long as the other tissues in the chamber.

No attempt has been made at this time to give a complete account of the processes involved in either the laying down or the absorption of this bony tissue. The present article aims chiefly to demonstrate a new method for the study of bone, and to show, with the aid of camera-lucida drawings, the feasibility of making such a study.

The tissue which precedes the first formation of bone has an appearance very similar to that of connective tissue, and it develops in regions in which the latter is present. However, a difference between the two can be observed, since the fibers of the typical connective tissue are distinct and very transparent, and are arranged, for the most part, in parallel bundles, while the fibers of the early bone tissue are less sharp in outline, less transparent, and are arranged in heavy bundles which run in many directions (fig. 1, *B*). These fibers are connected with the highly granular region of new bone (fig. 1, *C*). Lying between these heavy bundles are round and oval-shaped, very clear regions which vary greatly in size. The first change observed in watching such a new growing region is the extension of small, highly refractile granules into the bundles from the highly granular region, *C*. The bundles gradually become a mass of closely packed granules (illustrated at the top of the scale of figure 2) which encroach upon the clear, oval, and round regions to the extent of displacing some of them and narrowing others. Along with this encroachment there appears a highly refractile edge at the periphery of each oval or circular space. From day to day the early granules, which measure about $\frac{1}{2} \mu$ in diam-

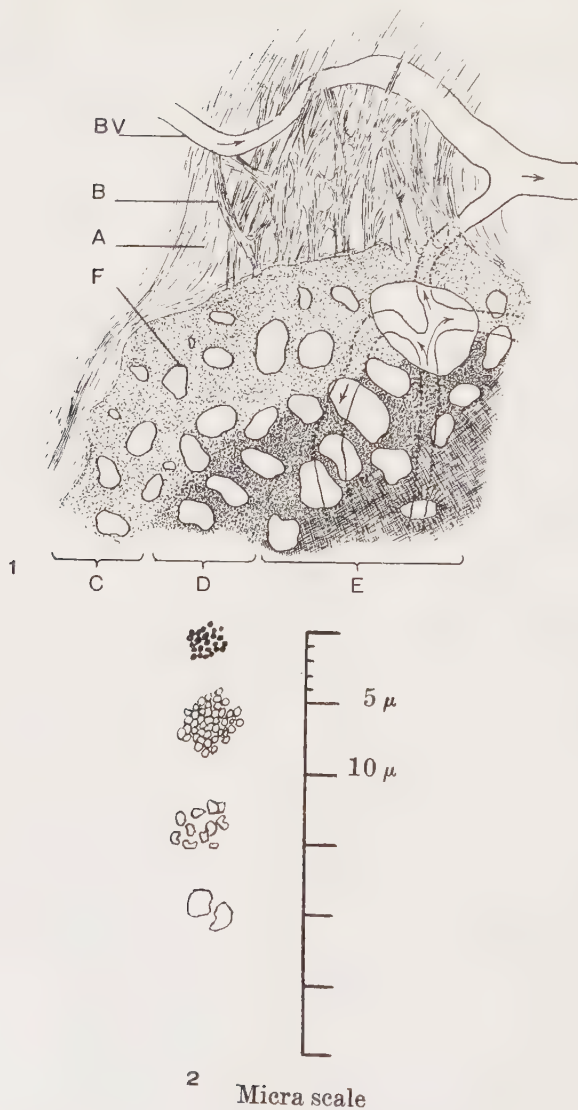


Fig. 1 A region showing the early formation of bone. *A*, connective tissue; *B*, one of the heavy bundles of fibers in which the earliest granules of bone formation appear; *C*, earliest granules; *D*, later granules; *E*, coalescence of granules; *F*, a lacunar space; *BV*, blood vessel. Camera lucida, $\times 335$. Granules shown diagrammatically.

Fig. 2 Size and shape of different types of granules in bone formation. Those at the top of the scale are the earliest ones to appear. Free-hand drawing, $\times 1900$.

eter, become fainter, larger, and more irregular in shape until finally they coalesce, thus forming a hyaline-appearing, homogeneous ground substance.

At present, it has not been determined what part the bone-forming cells, or osteoblasts, play in this development of the

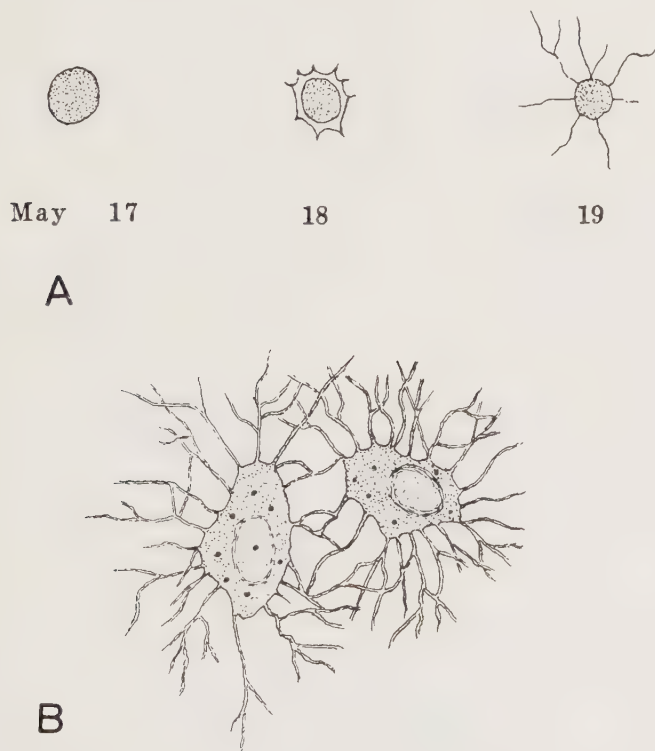


Fig. 3 A and B. A, showing appearance of a newly forming bone cell observed for three days, May 17th, 18th, and 19th. Stippling indicates high refraction of the cell. Camera lucida, $\times 670$. B, well-formed bone cells. A free-hand double enlargement of a camera-lucida drawing. $\times 1340$.

ground substance. Crawling on the cover-slip just above the new-forming bone and lying here and there in the clear spaces and among the fiber bundles, large, faintly granular, single-nucleated cells may be seen, which look not unlike macrophages. Some of these may very well be osteoblasts. The study of the development of the adult bone cells which lie in

their lacunae is likewise incomplete. It has been observed, however, that bone cells become visible in some of the round and oval clear spaces which have a highly refractile periphery



24 th



28 th



1 st

Fig. 4 Same bone cells observed for nine days. As seen on May 24th and 28th, and June 1st. Camera lucida, $\times 670$.

(fig. 3 A, *May 17*). At first, in the case of one cell observed (fig. 3 A, 18) a clear zone surrounded this refractile border which in time was surrounded by another refractile but

pointed border. The next day (19), the clear zone had disappeared and in the space or lacuna could be seen a bone cell with a scanty number of visible processes. Arising from this same cell, there gradually appeared more processes extending through the surrounding hyaline substance, where they presented a double-walled aspect due, evidently, to the bony wall of the canaliculi in which they lay (fig. 3 B).

The mature bone cells are easily recognizable. They are quite large and, depending upon the depth to which one

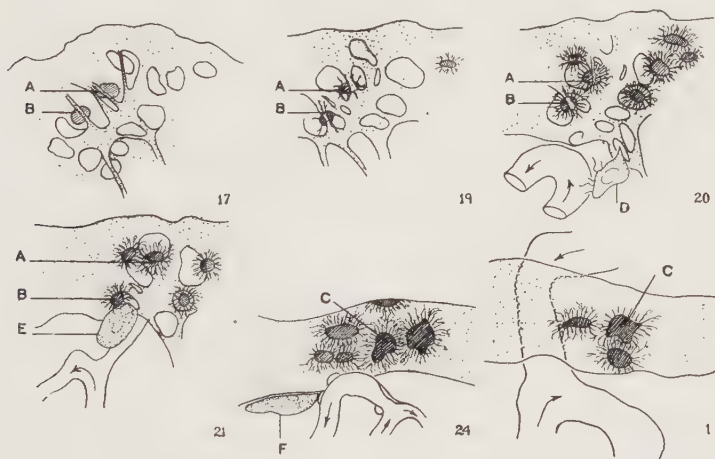


Fig. 5 The same region of new-forming bone over a period of sixteen days, as seen on May 17th, 19th, 20th, 21st, 24th, and June 1st. *A* and *B*, the same two bone cells seen for five days; *C*, the same bone cell seen for eight days; *D*, probably a macrophage; *E*, probably an osteoclast. Bone resorption is indicated. Outlines made with the camera lucida; appearance of the bone and the processes of the bone cells shown diagrammatically. $\times 223$.

focuses upon them, are either more refractile or less refractile than the ground substance in which they lie. Instead of a sharply outlined nucleus, they contain an irregularly shaped, dark circle. They vary in shape and do not occupy the same position from day to day (fig. 4). The great number of processes which they give off is very striking, and on account of their intertwining and anastomosis with each other and with those of neighboring bone cells, they have the appearance of a rich network of reticulum.



Fig. 6 Same region of bone with its blood supply over a period of fifteen days. As seen on May 18th, 20th, 21st, 22nd, 24th, and June 1st. *BC*, bone cells; stippling indicates bone matrix; *Art*, arteriole; *M*, a muscle cell of the arteriole; *Adv.*, adventitial (Rouget) cell of a capillary; *P.M.L.*, polymorphonuclear leucocyte 'sticking' to the endothelium; *Plat*, a circulating blood platelet surrounded by erythrocytes in a capillary; *Eryth*, extravasated erythrocytes; *Macro.*, a macrophage with digested erythrocytes; *Leuc.*, a wandering leucocyte; *A* is probably an osteoclast in a Howship's lacuna; *B* and *C* are also probably osteoclasts; *D*, a piece of bone undergoing absorption. The shading on drawing of May 24th indicates a new growth of bone on top of the old which fused with the latter by June 1st. Camera-lucida drawing with diagrammatical shading, $\times 223$.

In the same region where the bone is extending outward to form new bone, the process of resorption can also be observed (figs. 5 and 6). Typical Howship's lacunae which contain large multi- and singly nucleated cells appear throughout the tissue (fig. 6, *A*). A number of macrophages are also present in regions undergoing absorption.

DISCUSSION

It is hoped that enough has been presented to indicate the very great possibilities opened up by this new method for studying the microscopic details of bone growth and resorption by observation in the living. Prolonged observation should reveal many of the details at present lacking or incomplete, such as the manner of deposition of bone salts, the exact way in which the bone cells become surrounded by bone, and the fate of bone cells when released by resorption of the bone surrounding them; the process of bone resorption, including the origin of the osteoclast and the part played by it in resorption; the relation of the macrophage to the osteoclast and to bone resorption; the relation of bone growth and bone resorption to the action of stress and strain.

SUMMARY

A new method for the microscopic study of bone growth in the mammal by continuous observation in the living animal is described. The method consists in the placing of an autogenous transplant of bone marrow containing endosteum into the transparent chamber in the rabbit's ear previously described. New growth of bone from the transplant took place and preliminary observations on the new formation and absorption of bone have been carried out. The method makes possible the microscopic study of a wide variety of tissues by this method of transplantation into the transparent chamber inserted in the rabbit's ear.

I wish to express my gratitude to Dr. E. R. Clark, under whom these studies were made, for his stimulating interest in the development of this method for studying bone growth.

THE CIRCULATION OF THE NORMAL HUMAN KIDNEY

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The distribution of the arterial blood in the finer vessels of the kidney has been a point of discussion since shortly after Bowman's ('48) classical work on the relationship of the 'globules' of Malpighi to the arteries. The excellent investigations of Huber ('06), MacCallum ('26), and others have been largely confined to a study of the kidneys of various animals, such as the rabbit, guinea-pig, dog, and cat. Although this work is of inestimable value in the correlation of anatomic and physiologic data and the formulation of theories of urinary secretion, it is of the utmost importance to pathologists in investigating diseases of the kidney in man to have a clean-cut conception of the circulation of the human kidney.

Bowman's original paper figures injection of human glomeruli and in a footnote he mentions that he has taken every opportunity to study the process of Bright's disease in the human kidney. As Bowman is silent on any description of the human kidney separately from his general description, one would conclude that he considered that the circulation was essentially the same in all the kidneys studied. Bowman's results were unequivocally stated in the following words: "All the blood of the renal artery (with the exception of a small quantity distributed to the capsule, surrounding fat, and coats of the larger vessels) enters the capillary tufts of the Malpighian bodies; thence it passes into the capillary plexus surrounding the uriniferous tubes, and it finally leaves the organ through the branches of the renal vein."

The questions which have been discussed since Bowman's paper revolve about one point. Does all blood entering the kidney (except a small amount as nourishment to the structures of the hilus) pass through glomeruli and then to the tubules of the cortex and medulla? The negative answers to this question have postulated three possibilities: 1) that the interlobular arteries, after giving off the glomerular branches, proceed to the surface of the cortex where they break up into a capillary plexus continuous with the peritubular plexus of the cortex; 2) that small arteries arise from the under surface of the arcuate arteries and descend through the medulla as the arteriolae rectae verae; 3) that there are direct connections between the arteries and veins.

In addition to these questions concerning the general course of blood through the kidney, two special points are of interest: 1) the course of blood through the glomerulus from the afferent to efferent vessel, and 2) the distribution of the efferent vessel.

It is my intention to record in this paper observations made on human kidneys injected by various methods with a view of arriving at some definite statement regarding each of the points which have been raised.

LITERATURE

Virchow ('57), after numerous injections of human kidneys, arrived at very definite conclusions. He found that all branches of the renal arteries in the outer portion of the cortex ended in glomeruli. In the inner third he described a dual distribution, some vessels as afferent branches to the border-zone glomeruli and others directly to the medulla as arteriolae rectae verae. In addition to this origin of the arteriolae rectae, he described another origin, namely, a continuation of the cortical peritubular plexus into the medulla. Virchow thus definitely commits himself to the proposition that the kidney circulation is not a primary glomerular circulation.

Other investigators, notably Zondek ('01) and Golubew ('93), have recorded observations on injection of human kid-

neys. Golubew further complicated the question of the origin of the arteriolar rectae by postulating a triple origin: first, from the efferent vessel of glomeruli; second, as a branch from the afferent vessel just before it enters the glomeruli, and, third, from the larger arteries. He further postulated that there were direct connections between the arteries and veins. Zondek brought the question to a more exact conception, but still refused to acknowledge that there were not a few arteriolar rectae derived directly from the larger renal arteries.

Johnson ('99) reports observations on the circulation within a single glomerulus of the human kidney. He proves by modeling that the capillary channel is not one long continuous tube from vasa afferentia to vasa efferentia, but that there are numerous connections so that the entire minute-volume flow does not necessarily pass through the entire glomerulus.

More recently, Morison ('26) and Lee-Brown ('24) from Hinman's laboratory have recorded their observations on the human kidney. They record the presence of a rare arteriola recta, but offer a new conception for the significance of them. They suggest that in the corticomedullary region there are abortive and atrophic glomeruli in which the capillary loops may be so inconspicuous as to resemble an arteriola recta.

This year, Vimtrup ('28) has described the intraglomerular circulation in no uncertain terms as being devoid of anastomoses. By microdissection of injected specimens he concluded that a glomerular tuft consists of about fifty independent capillary loops.

TECHNIQUE

In studying this problem a great variety of injection methods have been used, not only to secure different pictures of the same thing, but also to have the observations of one type of injection serve as a check on others. Injections of normal human kidneys have been made, using gelatin carmine, Janus green, x-ray opaque media, celloidin corrosion, and aqueous Berlin blue. Consideration of the question of what

is normal has been based on gross observations of one kidney (the one injected) and gross and microscopic examination of the opposite kidney, together with observation of microscopic sections of the injected kidney whenever this was possible.

Only two points in connection with technique need be recorded here: first, the method of preparing gelatin carmine and second, the preparation of the celloidin solution for the corrosion method.

A great deal of difficulty was experienced in securing uniform lots of gelatin carmine. To obviate the human factor as much as possible, I have controlled the neutralization by the hydrogen electrode. Using the method of Mallory and Wright ('24), 80 grams of gelatin and 20 grams of potassium iodide are dissolved in 200 cc. of water. To this is added 15 grams of carmine suspended in 100 cc. of water to which has been added sufficient ammonia to dissolve the carmine. After thorough mixing, a freshly platinized electrode is immersed in this mixture and readings of voltage made in the same manner as in hydrogen-ion concentration measurements. Acetic acid is cautiously added with thorough stirring until the voltage corresponds to a pH of 7.2. The media is now ready for injection. Using this method, I have secured consistently good results.

For the celloidin medium a 2.75 per cent solution of parlodion and 2 per cent camphor in acetone has been used. In addition to determining the strength of the celloidin by weight, I have controlled the final product by viscosity measurements. By experiment it was found that a solution with a relative viscosity of 18 (at 20° referred to water) was of such strength that it would pass to the glomeruli, but not beyond, in the human kidney. By increasing the viscosity to 20, the glomeruli are not injected and by decreasing it to 15, the postglomerular vessels and veins are injected. By this method one has a very accurate control to secure any degree of injection desired and thus study the vascular tree in parts. A further discussion of the principles and applications of injection methods is given by Scott and Moore ('27).

The general technique of injection may be briefly stated as follows: The kidney is removed from the body as soon as possible after death, and a cannula inserted into the renal artery. Warm saline is passed through the capillary bed until inspection of the surface reveals a uniform white to gray color. Air is now passed through with the kidney immersed in water and all cut vessels ligated, leaving open only the main-stem artery and vein. A pressure of 100 to 200 mm. of Hg is used in these two processes. A bottle of injection mass is now connected to the cannula and injected under a pressure of 600 mm. of Hg. For details of the celloidin injection, see Moore and Scott ('27) and for the gelatin carmine, see Moore ('28).

In the reliability of results, there is no doubt that the combination of Janus green and gelatin carmine as given by MacCallum ('26) gives the most accurate pictures and the best preparations for study. Not only is one able to differentiate artery and vein, but also to detect defects of injection not seen in other methods. The celloidin corrosion method is of value in a study of the corroded specimen and also for study of the relations of the vessels to the kidney substance by partial maceration and dissection. X-ray opaque methods are of little value in following finer circulation and Berlin blue offers no advantages over gelatin carmine.

PERSONAL OBSERVATIONS

Although the distribution of the larger vessels is well known, it seems advisable to briefly review the observations which have been made in this laboratory on human kidneys. The manner in which the renal artery divides into its primary branches is so variable and deviates so markedly from the typical anterior and posterior branches described in textbooks of anatomy that we are accumulating additional data which will be published in a separate communication. As the primary branches of the renal artery enter the hilus of the kidney and pass around the pelvis, they divide into as many secondary branches as there are papillae in the kidney. The

term lobar artery seems appropriate for these vessels. If a kidney is prepared for injection by the usual method and one of these lobar arteries ligated just before the mass is injected, the portion of kidney supplied by this ligated vessel does not inject, except for a variable thin area just beneath the capsule, which shows that the lobar arteries are the fundamental vascular units of the kidney and that they do not anastomose with adjoining vascular units.

As each lobar artery approaches the papillae it divides dichotomously into two (sometimes three) vessels which pass toward the cortex on each side of the pyramid. These are the interlobar arteries and by injection of solid particles it can be proved quite conclusively that these vessels also do not anastomose with contiguous arteries. As these vessels pass along the sides of the pyramid, branches are given off at right angles which pass around the pyramid and also upward to the cortex.

When the interlobar arteries reach the corticomedullary junction, they divide dichotomously, each branch passing at right angles to the parent stem and lying in the midsagittal plane of the kidney. The point of interest is that these vessels, the so-called 'arcuate arteries,' do not pass on to meet the arcuate from the adjoining lobe, but terminate just short of the midpoint between the two lobes. From the arcuate arteries, vessels arise in two planes, some pass upward through the cortex in a sagittal plane as the interlobular arteries, while others pass around the kidney in a horizontal plane. These vessels in turn give rise to interlobular arteries. Up to this point the relations may be followed by careful gross dissection, but beyond this, recourse must be had to the microscope, at least for confirmation of gross findings.

The first point to be carefully checked by microscopic examination is the presence or absence of small branches of the vessels already outlined up to and including the arcuate arteries and, if present, what is their ultimate distribution. After a study of many miscellaneous sections and a complete study of serial sections of an entire kidney (each section 75μ thick,

from an eight-year-old boy), I am prepared to make the positive statement that I have not seen a single vessel of any size pass from a lobar, interlobar, or arcuate artery to the parenchyma of the kidney without first passing through a glomerulus. This statement carries with it the interpretation that there are no arteriolae rectae arising from the arcuate arteries directly.

Passing on to the interlobular arteries, the ultimate distribution of their branches is the next point of investigation. The answer to this question is that I have not seen any branch of an interlobular artery which did not pass directly to a glomerulus, except in a few instances. These exceptions are open to some doubt. Ordinary inspection under low magnification of injections gives a picture in many instances of branches of the interlobulars direct to the peritubular plexus. High-power observation of these same fields leaves no doubt in the majority of cases that these apparent branches are only lying on top of or under the larger vessels. Further, thin sections (10 to 15 μ) fail to reveal this apparent branching. Despite the most careful observation, I have been unable to prove in several instances that there are not a few small branches of the interlobular arteries directly to the peritubular plexus, but this finding is most certainly the exceptional thing and the scarcity of this type of vessel can have little bearing on the ultimate distribution of arterial blood throughout the kidney.

The vessels having been traced to the vasa afferentia of the glomeruli, the actual course through the individual glomeruli is of interest, in view of Johnson's ('99) investigations. I have not attempted to trace the course of capillary plexus of the glomeruli, but only by indirect evidence confirm Johnson's findings. This indirect evidence is that in some normal kidneys and in practically all cases of glomerulonephritis, numerous glomeruli may be seen in which one or two lobes are perfectly injected and one lobe not injected at all. The interpretation of this observation, in view of the conflicting views of Johnson and Vimtrup, is difficult, and judgment will

be withheld until a later date when our observations on the total number of glomeruli in animals and man are completed. At present we are inclined to accept Vimtrup's explanation of independent capillary loops without anastomoses as the most logical.

The distribution of the vasa efferentia as they leave the glomeruli is variable. In the case of the border-zone glomeruli, many but not all, pass directly into the medulla as the arteriolae rectae. The remainder in the border zone and all in the outer cortex break up into the peritubular plexus. The question of the exact distribution of each vas efferens is of great importance in any theory of urinary secretion. Certain indirect evidence is available to answer this question. In many kidneys in which the injection has been incomplete, one glomerulus may be injected and all surrounding glomeruli not injected. Because of the high pressure used (600 mm. Hg) and because of the high degree of obstruction in the capillaries, many of the injected glomeruli rupture and fill the proximal convoluted tubules with gelatin carmine. Under this combination of favorable circumstances, I have observed many injected glomeruli and tubules with the peritubular plexus injected only about the tubules containing carmine mass. Although undoubtedly a certain amount of anastomosis takes place between adjoining secretion units, I believe that in general we may say that the vas efferens of a glomerulus is distributed to the peritubular plexus surrounding the tubules of that glomerulus.

The blood from the peritubular plexus is returned to the interlobular veins in part, but in many kidneys, especially those from children less than two years of age where the differentiation of pars convoluta and pars radiata is marked, direct extension of the peritubular plexus into the pars radiata and thence down into the medulla as arteriolae rectae spuriae is quite well shown.

It may be added that I have not observed any direct connection between the arteries and veins such as postulated by Golubew ('93). This observation is further checked by the

fact that when an injection mass of viscosity greater than 20 is injected into the artery, no veins are injected, and further, the medulla remains absolutely uninjected, proving that a fluid to pass either into the veins or the arteriolae rectae must be of a viscosity capable of flowing through the vasa efferentia of the glomeruli.

In some human kidneys there are branches of the arcuate arteries which pass directly through the cortex to supply the capsule and anastomose with vessels there. In the cases in which I have had the opportunity to study the vessels, they are not continuations of the interlobulars, but are separate vessels which ascend through the cortex without giving any branches to the parenchyma. As to the ability of these vessels to carry on a collateral circulation in case of occlusion of the renal artery or one of its branches, I have no direct evidence, but from their size certainly one would conclude that such a collateral circulation is possible.

SUMMARY

1. The portion of kidney drained by a papilla is the vascular unit of the kidney.
2. All vessels of the kidney from the renal artery itself to and including the afferent vessels of the glomeruli are of the type of arteries which do not anastomose with adjacent vessels to the extent of preventing an ischaemic necrosis of the tissue beyond in case they are occluded.
3. The vas efferens of a glomerulus is distributed, in general, to the tubules of that glomerulus.
4. All blood entering the kidney, except that distributed to the hilus structures and possibly a few small vessels to the cortex, passes through a glomerulus before entering the peritubular plexus or the arteriolae rectae. Hence, the kidney circulation is primarily a glomerular circulation.
5. The arteriolae rectae have a double origin, in part from the vasa efferentia of the border-zone glomeruli, and in part as continuation of the vessels of the pars radiata. Under no circumstances has an arteriola recta been seen arising from

a vessel the blood of which has not previously passed through a glomerulus.

6. There are no direct connections between the arteries and veins.

7. The arcuate arteries of one unit do not anastomose with the arcuate arteries of the adjoining vascular units.

8. In some human kidneys there are direct branches of the arcuate arteries which pass through the cortex to the perinephritic tissue.

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THE UTRICULO-ENDOLYMPHATIC VALVE¹

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ONE HELIOTYPE PLATE AND TWO TEXT FIGURES (FOUR FIGURES)

This description of a structure which, for the want of a better name, I have called the utriculo-endolymphatic valve, is based on the study of a large number of serial sections and reconstructions of human fetuses. Of the twenty-five fetal ears which were sectioned, fourteen were received from Dr. George L. Streeter. Another, of a 183-mm. human fetus, was furnished by Dr. George W. Bartelmez. This was especially well preserved and the illustrations in this paper are all taken from it. The rest of the embryos were collected at Madison, Wisconsin.

In introducing the term utriculo-endolymphatic valve, the writer wishes to make clear that it is used in this paper as an anatomical term and does not carry physiological implications. Anatomically, the structure resembles a cusp or valve, but this does not necessarily mean that it is a functional valve. The function of this structure has not been determined.

In appearance it is a highly cellular connective-tissue valve-like flap lined by columnar epithelium. It projects into the utriculus and guards the utricular opening into the utriculo-endolymphatic duct. This valve-like structure is a fold of the posterior wall of the utriculus and utriculo-endolymphatic duct wall at the point of junction. Figure 4 (*UV*) shows the lower half of the valve and figure 2 (*UV*) is a photomicrograph of a section through the middle of the valve.

¹This report is one of the by-products of an investigation on the development of the capsule of the human ear sponsored by The American Otological Society.

The utriculo-endolymphatic duct (*UD*, figs. 2, 3, 4) is a small duct whose lumen measures about 100μ in diameter. As it enters the utricle it flattens postero-anteriorly and

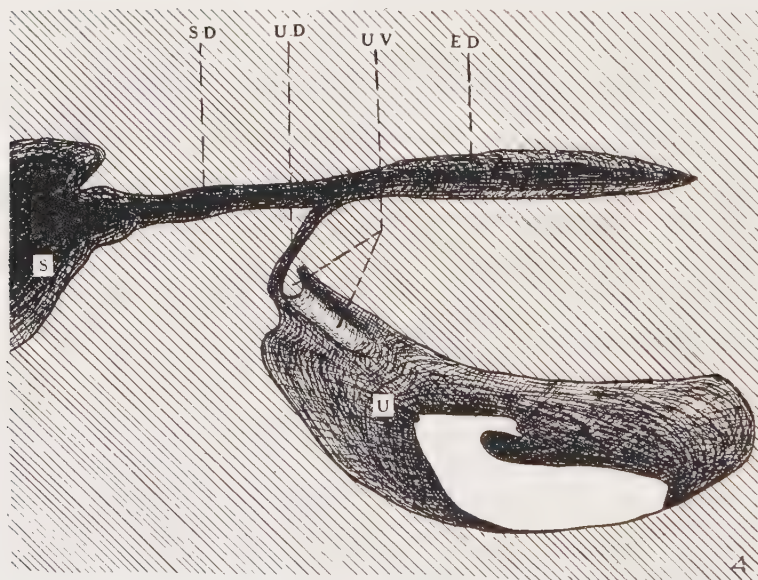
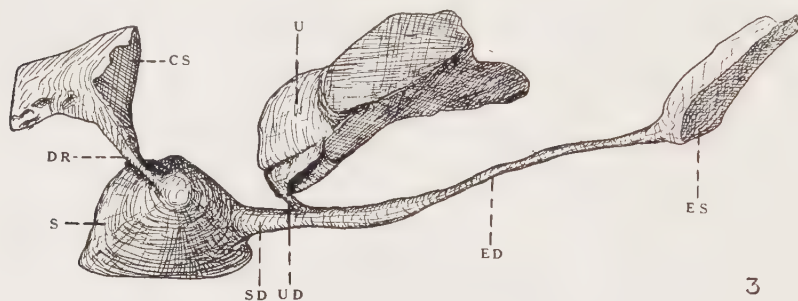


Fig. 3 Drawing of a model of part of the endolymphatic system of a 183-mm. (crown-rump length) human fetus.

Fig. 4 Drawing of a negative model of part of the model shown in figure 3. *S*, sacculus; *U*, utricle; *ES*, endolymphatic sac; *ED*, endolymphatic duct; *UD*, utriculo-endolymphatic duct; *SD*, sacculo-endolymphatic duct; *UV*, utriculo-endolymphatic valve; *DR*, ductus reuniens; *CS*, cul de sac.

spreads in a fantail manner, as shown in figure 3. Its opening into the utricle is slit-like, as shown in figure 4. This slit-like opening is guarded by the utriculo-endolymphatic valve (*UV*), as seen in figure 4.

Histologically, the valve consists of a highly cellular connective tissue which is lined by columnar epithelium, as shown in figure 2 (*UV*). This highly cellular tissue is a continuation of the paralympathic tissue surrounding the endolymph system (fig. 2). I have been unable to determine from the fetal material the exact nature of the cells. They look like connective cells but may be myoblasts. Because all of my preparations are cut in series and stained in hematoxylin and eosin, I have not had the opportunity to make special stains. There is, therefore, a possibility that smooth-muscle fibers are present. There is, however, no indication of nerve fibers coming into this region.

DISCUSSION

As far as we know, there is no previous account of this valve-like structure. The structure, when followed out in serial sections and when reconstructed, is so definite that it is hard to believe that it should have been overlooked so long. The only explanation for this may lie in the fact that the walls of the endolymphatic structures are so delicate that they are easily distorted or torn. The writer overlooked the structure for a long time, considering it an artifact until preparations were obtained in which the structures were so well preserved that its existence could not be doubted. We have followed it in all of our human fetal ears and also in pig ears.

It seems, therefore, that a structure so definite as this valve-like structure should have a definite function. So far there is no experimental evidence suggesting its significance. Therefore, whatever is said at this time regarding its function is merely conjecture. In the abstract of the paper read before the American Association of Anatomists at the Ann Arbor meeting (*Anat. Rec.*, vol. 38, p. 3) the following sug-

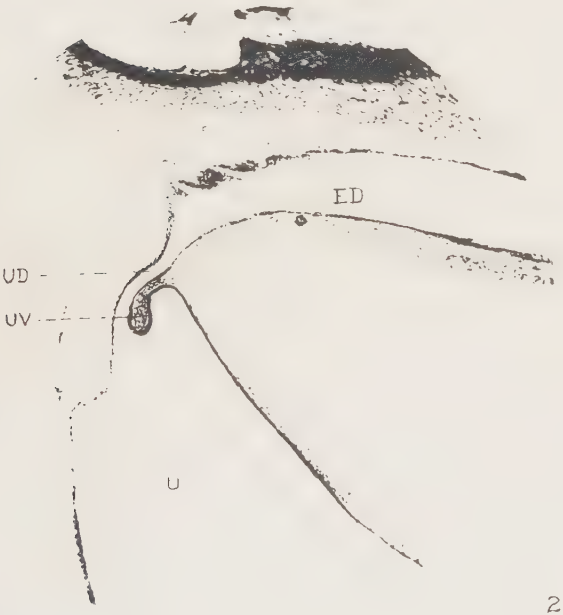
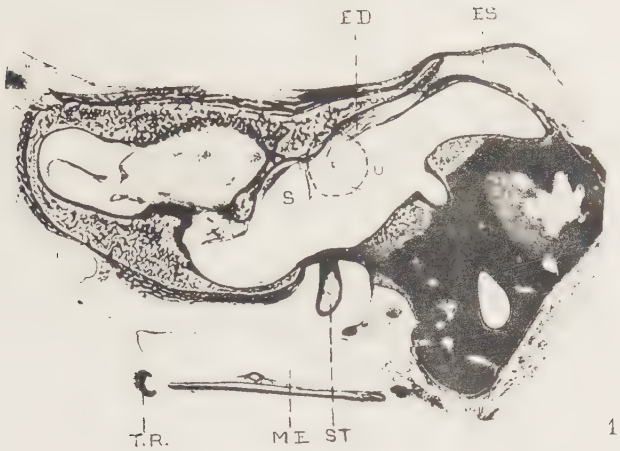
gestion was made as to the possible function of the valve: "Its position would indicate that the flow of endolymph is from the endolymphatic duct to the utricle, but this is not a necessary conclusion. If the flow is in the other direction, the slow movement of the endolymph may not affect this valve. On the other hand, in case of any sudden pressure disturbance this valve may prevent the outflow of endolymph from the utricle, thus maintaining a more constant pressure within the utricle and semicircular canals." It is important that experimental procedures should be devised to test such a hypothesis.

PLATE 1

EXPLANATION OF FIGURES

1 Photomicrograph of section through the labyrinthine capsule of a 183-mm. (crown-rump length) human fetus. Magnification about four diameters.

2 High-power photomicrograph of the area indicated in figure 1 by circle, showing a section through the middle of the utriculo-endolymphatic valve, the utriculo-endolymphatic duct, the endolymphatic duct, and utriculus. *ST*, stapes; *ME*, middle ear; *TR*, tympanic ring; *S*, saccule; *U*, utricle; *ED*, endolymphatic duct; *ES*, endolymphatic sac; *UD*, utriculo-endolymphatic duct; *UV*, utriculo-endolymphatic valve.



THE DEVELOPMENTAL POTENCIES OF THE INTER-MEDIATE MESODERM OF AMBLYSTOMA WHEN TRANSPLANTED INTO VENTROLATERAL SITES IN OTHER EMBRYOS: THE PRIMORDIAL GERM CELLS OF SUCH GRAFTS AND THEIR RÔLE IN THE DEVELOPMENT OF A GONAD

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ONE TEXT FIGURE AND FOUR HELIOTYPE PLATES (TWENTY-FOUR FIGURES)

INTRODUCTION

In a previous paper (Humphrey, '27) it was shown that complete unilateral extirpation of the primordial germ cells of Amblystoma embryos in stages 32 to 36 (Harrison's standard stages) resulted in the absence of a normal gonad on the operated side, in animals surviving as long as 300 days. The operation for removal of the primordial germ cells of course necessitated the disturbance or destruction of a certain amount of adjacent embryonic material, since the lateral portion of the axial mesoderm, the wolffian duct, and the medial portion of the lateral mesoderm, together with the overlying ectoderm, were removed along with that intermediate mesoderm containing the primordial germ cells or their forerunners. That the absence of the gonad following an operation of this character might be interpreted as due to growth disturbances rather than to removal of any necessary formative elements was considered in the previous report, in which, however, it was concluded that the absence of the gonad was a direct result of the elimination of the primordial germ cells, together possibly with related mesoderm.

To test the developmental potencies of the embryonic tissues removed in the preceding experiments (Humphrey, '27, figs. 1 and 2), these have now been transplanted into the lateral body wall of another embryo, and allowed to develop for periods up to seventy days. These grafts show beyond question that certain cells of the mesoderm of *Amblystoma*, from a very early period of embryonic life, possess the capacity for differentiation into primordial germ cells and that such cells, if favorably situated in the graft and if present in sufficient numbers, may give rise to a gonad. The results of the present series of experiments therefore support the conclusions reached in the previous report and give valuable data as to the early specificity of the genital preprimordia in *Amblystoma*, a specificity acquired long before definite anlagen of the gonads (in the form of genital ridges) have made their appearance.

MATERIAL AND TECHNIQUE

Amblystoma embryos of stages 21 to 34 were used in these experiments. In embryos of the more advanced stages the area removed for transplantation consisted of a strip extending in a caudal direction from the level of the ninth or tenth somite to that of the sixteenth or seventeenth somite (fig. 1). In younger embryos with fewer than ten somites developed, it was necessary to estimate the approximate position of the tenth somite and to remove a strip extending from this level back to a point close to the blastoporic margin. In all cases the strip removed for transplantation included the lateral border of the axial mesoderm, the intermediate mesoderm (nephrogenic material and germ cells or their fore-runners), and the medial border of the adjacent lateral mesoderm. In the younger embryos this mesodermal material, when removed, was yet in the form of two simple, closely approximated sheets in which the regions mentioned above could be localized only approximately even in sections of fixed material. In older stages the somites were of course developed, and in consequence the various subdivisions of

the mesoderm were more sharply marked. In older embryos a portion of the wolffian duct also was removed in the transplant. In all cases the mesodermal material was removed and transplanted attached to the overlying ectoderm. Usually, the inner layer of the mesoderm was not strongly adherent to the entoderm, peeling off from this layer quite readily. The transplants never included entodermal cells in any case, since if the mesoderm and entoderm failed to

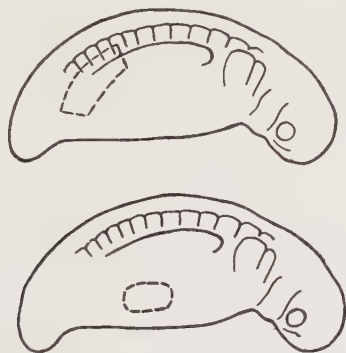


Fig. 1 Above, an *Amblystoma* embryo of stage 29, with the area removed for transplantation outlined in broken lines. This area extends from the level of the ninth or tenth somite to that of the sixteenth or seventeenth. It includes the intermediate mesoderm (mesonephric and gonadal preprimordia) and the adjacent portions of the axial and lateral mesoderm, together with the overlying ectoderm.

Below, an embryo of the same stage, showing (in broken lines) the site into which the graft is implanted. The ectoderm and mesoderm are removed from the area outlined and the entoderm excavated to form a shallow pit for the reception of the transplant.

separate easily, mesodermal cells were left adhering to the entoderm, but never the reverse.

To prepare the host for the reception of the transplant described above, a small area of ectoderm and mesoderm was removed from the lateral part of the body at some point between the level of the third or fourth somite and the blastopore or anal opening (fig. 1). The entoderm at this point was usually excavated also, to form a shallow pit. Into the site thus prepared a transplant was brought from another

embryo in the same operating dish, placed in position with needles, and held in place by suitable glass rods for from one to several hours, until it had firmly healed into its new position. The donor of a transplant and the host embryo or recipient were then transferred into the same finger bowl and thus kept together, unless their unequal growth made it necessary to separate them, in which case the experiment number was still kept on each of the separate dishes. In this way the development of structures in the graft could readily be checked with the absence of organs or structures in the donor embryo, and the sex of the donor could be accurately determined, a point of importance in considering the effect of the host upon sex differentiation in the graft gonad.

Two species of *Amblystoma* are obtainable locally at Buffalo and embryos of both were utilized. In over half the total number of operations the transplant was from an *Amblystoma jeffersonianum* donor into a host of the same species and approximately the same stage. This species was used in greater numbers in preference to *Amblystoma maculatum* because of the greater number of primordial germ cells it has been found to possess.

Of the remaining transplantations, about half were from *Amblystoma maculatum* donors into hosts of the same species and stage of development, while the remainder were from *Amblystoma jeffersonianum* donors into *punctatum* hosts which usually differed by several stages from the development of the donor embryo. Little difficulty was found in securing a successful union of the transplant with the host germ layers in any of the types of operation mentioned above.

In all, 180 transplants were made in the manner described above. Out of this number only forty-nine pairs (donor and host, as in fig. 1) were reared to fifty days or more. If the host died, the corresponding donor was either discarded or fixed; if the donor died, the host was either fixed for study of the early stages of transplant development or kept until

fifty to seventy days after operation, at which time all hosts were killed and fixed. From the death of one or the other member, the total number of donor-host pairs was reduced to a rather low figure (27 per cent of the total number of operations). That this, however, does not indicate an actual mortality of 73 per cent may be seen from the fact that twenty-five additional hosts were reared past fifty days, after the corresponding donor had died at some earlier date, while several more hosts were killed in younger stages.

The highest percentage of deaths in the hosts occurred within ten days after implantation of the graft. That many such deaths were a direct result of the operation, was indicated by hernia or necrosis at the site of the transplant. Other deaths, however, resulted from interference with normal developmental processes in vital organs. Some embryos, for example, died as a result of defective development of the circulatory system, while others, in older stages, died as a result of apparent inability to feed. The death rate among the donor embryos was approximately the same as that of the hosts.

Host embryos and larvae of younger stages were fixed entire in Bouin's fluid, after which the region of the body containing the graft was serially sectioned and stained with Meyer's hematoxylin and eosin. Older animals were killed and placed in Bouin's fluid, in which the body cavity was opened, and the graft examined and sketched with the aid of a binocular microscope. The graft with the area of body wall immediately surrounding it was then removed, embedded, and prepared for study as in the younger animals. The gonads of the host were also removed (attached to the mesonephroi) and sectioned transversely. The donor, usually killed at the same time as the corresponding host, was likewise dissected and studied in Bouin's fluid under the binocular microscope before removal of the gonad and related structures for sectioning.

POSITION AND RELATIONS OF GRAFT

Although the graft was implanted in a lateral position, growth of the surrounding tissues of the host usually shifted it to a more ventral location (fig. 3). Often in animals of fifty to seventy days it was very close to a midventral situation. Only occasionally was the transplant carried dorsally in such fashion that its derivatives could fuse with those of the intermediate mesoderm of the host. Some few of the grafts in older larvae were found close in front of the pelvic girdle and some few were equally close to the pectoral region, but the great majority were in the middle of the body, commonly not far from the caudal border of the liver (fig. 2).

When implanted, the innermost layer of the graft was in direct contact with the entoderm of the host, whose own ectoderm and mesoderm had been stripped from the site prepared to receive the transplant. The ectoderm of the host united with the ectoderm of the transplant to form a continuous layer, while the mesoderm of the host fused with the mesodermal layers of the graft. With the formation of the coelomic cavity, however, the bulk of the mesodermal material of the graft commonly remained attached to or included in the somatopleure of the host. In embryos in which the coelom has but recently appeared, a broad strand or bridge of mesodermal cells frequently extends from the somatopleure to the gut (figs. 15 and 18). This is probably broken in a great many cases; at any rate, the gut often exhibits no distinguishable modification to mark its early relation to the graft. In over half the older animals, however, the mesodermal connection of earlier stages is found represented by a slender strand or cord of tissue joining the graft site to the intestine. This strand is generally 5 mm. or less in length and frequently carries a blood vessel of relatively large size. Due to elongation and rotation of the gut, the strand may become extended to a length of 10 or 12 mm. In such cases it is usually very slender and without patent blood vessels. This strand consists chiefly of connective tissue with its surface covered by mesothelium pro-

longed over it from the body wall or gut. In many animals it attaches directly to the body wall; in others it is connected to one or another of the structures derived from the transplant (mesonephros, fat body, or gonad). It attaches to the intestine, usually, without marked enlargement. In hosts of fifty days or older no recognizable derivatives of the graft mesoderm (mesonephros, gonad, or fat body) are usually to be found on the intestine at the point of attachment of the strand of tissue above described. In two younger hosts, fixed seventeen days after grafting, groups of germ cells were found on the gut.

Frequently, the site of the graft is marked externally by a prominent elevation, as shown in figure 2; in such animals, at autopsy, it is usually found that on the inside a depression or pit occupies a corresponding position; and attached to the sides or bottom of this pit are various graft derivatives.

MUSCLE AND CARTILAGE OF GRAFT

In examining grafts fixed within a few days after operation it is found that the transplanted axial mesoderm has continued differentiation and has given rise to embryonic skeletal-muscle fibers (fig. 16). Although these muscle fibers are responsible for the prominence of the graft in early stages, in older embryos they are frequently found undergoing degeneration. In the grafts removed from still older specimens the muscular layer of the somatopleure is usually thinner at the graft site than elsewhere, the prominence of this region now being due to the increased size of other graft derivatives, generally the mesonephros. Small muscle fibers of atrophic appearance found associated with other graft derivatives in hosts fifty days of age or over may possibly be persistent fibers derived from implanted myotomic material.

Of seventy-four grafts removed fifty days or more after implantation, twenty-five contain one or more nodules of cartilage. As a rule, this cartilage constitutes but a small proportion of the total bulk of the graft derivatives (figs.

5 and 6). Ordinarily, it is in the form of a single nodule or flattened plate, located in the body wall in close relation to other graft derivatives. In one animal such a cartilaginous plate was found undergoing replacement by osseous tissue.

The cartilage encountered in these grafts is interpreted as developing from material belonging to the preprimordium of the pelvic girdle or hind limb. This interpretation is supported by the fact that donors occasionally display defects of the hind limb on the side from which material has been removed to be used as a transplant. Such donors may show reduplication of the hind limb, an increase in the number of its digits, a reduction in the size of the limb, or even complete removal of the limb-bud or girdle material. In only one case, however, was a limb developed from a transplant; in all others the skeletal structures produced are of limited bulk and remain included in the tissues of the body wall.

The occurrence of cartilage is not limited to grafts taken from donors at any particular stage. Cartilage is present in one graft from a donor of stage 22 and in another from a donor of stage 33.

MESONEPHROS OF GRAFT

The intermediate mesoderm of the transplant differentiates into mesonephric tubules in a high percentage of cases, these structures being found in sixty-seven of seventy-four grafts fixed fifty days or more after implantation. Since mesonephric tubules are almost invariably present in the grafts studied after shorter periods of development, it is possible that their occasional absence in older grafts may sometimes be due to their having undergone degeneration and absorption.

The mesonephric tubules of the graft are ordinarily grouped in a compact mass. In the majority of cases this mass is somewhat flattened or disc-like in form and broadly attached to the body wall, frequently along one side or at the bottom of a distinct pit (fig. 5). In some few specimens the mesonephric mass, though flattened or disc-like, is at-

tached only by one edge; in other cases its connection to the body wall is reduced to a short slender stalk, as shown in figure 4. In some grafts, possibly because not subjected to pressure from the abdominal viscera of the host, the mesonephric tubules are in the form of a rounded mass, either broadly attached or stalked. In a few cases in which only a small number of tubules are present these tubules lie in the body wall surrounded by other tissues, and were not detected until the graft was sectioned.

Histologically, the mesonephroi of the grafts show many features characteristic of the normal. Nephrostomes are encountered occasionally (fig. 24). Glomeruli may be identified in about 85 per cent of the grafts (fig. 7). In about an equal percentage are tubules showing epithelium characteristic of the functional mesonephros of the host, as illustrated in figure 8. That the mesonephros of the graft was in many cases functionally active is indicated by the accumulation of fluid in the wolffian duct or the more distal portion of the mesonephric tubules themselves, producing marked dilatation of these structures or even converting them into small cysts. Cysts of large size are but rarely encountered, suggesting that reabsorption of fluid from the enlarged tubules eventually balanced its production. Distended tubules or cysts are found only in mesonephroi in which glomeruli are present, accompanied by segments of tubules showing a more or less normal epithelium. In mesonephroi with no glomeruli and all tubules of embryonic or atrophic appearance, enlarged tubules or cysts are invariably lacking.

Mesonephric tubules and glomeruli are found in grafts taken from donors as young as stage 22. The number of grafts from younger donors is insufficient to determine at what stage the mesonephric preprimordium becomes definitely established. Hoadley ('26 a, p. 213) states that in grafts of chick blastoderms, nephric structures are found only in cases in which the embryo yielding the transplant had an age of at least four hours of incubation at the time of the operation. He further states that "grafts of four-

hour blastoderms showed the development of only the secretory tubules of the kidney," while "if portions of the six-hour blastoderm are transplanted, the grafts show the development of the secretory tubules and the glomeruli." The four- or six-hour chick blastoderm represents a much earlier stage of development than does the *Amblystoma* embryo of stage 22, in which the first few somites have already appeared. It is not surprising, then, that in grafts taken from *Amblystomae* of stage 22 both tubules and glomeruli develop. The preprimordium of the mesonephros, undoubtedly, is already segregated in embryos considerably younger than any donors used; in embryos of stage 22, therefore, the units of this preprimordial segregate are able to differentiate specifically when transplanted into the body wall of another embryo. The development and differentiation of the mesonephric tubules in no way is dependent upon the presence of the wolffian duct, which was not included in grafts from the donors of earliest stages. Nor does the position of the graft in relation to the host mesonephros determine the development of its nephric constituents, since these may be as well differentiated in grafts recovered from a mid-ventral situation as in those more dorsally placed (i.e., closer to the mesonephros of the host).

FAT BODY OF GRAFT

Fat cells are found in half of the total numbers of grafts. Sometimes, only a few cells are present, spread out along the peritoneal surface, frequently along a blood vessel. More often, however, a considerable number of the cells are aggregated to form a distinct fat body (*corpus adiposum*; see fig. 12). This shows great variability in its relations to other structures of the graft. It may occur as an enlargement on the strand of tissue running from graft to gut, always close to the attachment of this strand to the graft; it may be attached either by one edge or by a stalk to the body wall or the surface of the mesonephros; more rarely, it is located in the ventral mesentery, as a rather thin sheet of

cells. In view of the normal mode of origin of the corpus adiposum in urodeles and its intimate relation to the gonad it is of interest to consider the relation of this body to the gonad of the graft.

In an examination of seventy-four grafts it was found that in twenty-five both gonad and fat body are present; in fifteen a gonad is present, but no fat body; in eleven a fat body is present but no gonad, and in twenty neither fat body nor gonad is to be found. It would thus appear that gonad and fat body are as often found together as either is found alone. Nevertheless, in the majority of cases in which the two structures are both present in a graft, they are not at all intimately related. They are usually attached separately to either body wall, mesonephros, or the strand running to the gut. In eight of twenty-five cases their attachments are fairly close together; in one case a slender common stalk branches to give short stalks to each of the two organs. In three cases the gonad is joined to the fat body by one edge, in two of these cases having no further attachment whatever to other tissues of the graft or host. From the eleven cases in which a fat body is present in the absence of a gonad and from the frequent lack of intimate relation between the two when both are present, it may be judged that the fat body is able to differentiate quite independently of the gonad, though closely associated with it in normal development. Previous extirpation experiments of the writer also show that a fat body may develop in the absence of a gonad (Humphrey, '27).

A fat body has been found in grafts from donors as young as stage 22. No grafts from younger donors developed to a stage at which a fat body might have been differentiated.

PRIMORDIAL GERM CELLS AND GONAD

In forty out of the seventy-four grafts allowed to develop for fifty to seventy days, a distinct structure which may be termed a gonad is present. In some cases this contains but a very few germ cells, while in others as many as several

hundred are present. In no case, however, does the graft gonad equal in size the normal organ of the host.

The gonad is highly variable in its relations to other structures of the graft. In fifteen cases of the forty it is attached by one edge or by a slender stalk to the surface of the mesonephros of the graft. In eleven cases it has a similar attachment to the body wall, frequently close to the mesonephros. In nine cases it has a stalked attachment to the strand joining graft and gut, while in two cases it occurs as an enlargement on this strand rather than as a stalked nodule. In the three remaining cases the gonad is attached to the fat body, as in figure 10.

The shape of the gonad depends somewhat upon its mode of attachment and the pressure exerted upon it by neighboring structures. Most often, when stalked, it is either pear-shaped or slightly flattened or disc-like (figs. 9 and 14). Usually, its longest diameter is not more than two or three times the length of its shortest; it never appears as a ridge equal in length to a normal gonad.

In its finer structure the gonad of the graft frequently shows the usual features of the normal gonad. If of ovarian type, as in figure 9, a distinct ovarian cavity may be present, with the lining epithelium characteristic of the normal ovary; surrounding this cavity and at the surface of the gonad are oogonia and oocytes in various stages of development. If, on the other hand, the gonad is a testis, no central cavity is developed; the so-called sex cords (anlagen of duct system) appear as compact groups of cells between the spermatogonia and the latter have a more uniform distribution through the organ (figs. 12 and 13). If a testis is attached to the surface of the mesonephros, it is sometimes possible to trace a duct-like cord of cells extending from the sex cords into the mesonephros; such connections correspond to the efferent ductules of the normal testis. In other cases, in which the relation of gonad and mesonephros is less intimate, it is impossible to find any such connection between them; the gonad may, nevertheless, have the duct system or ovarial

sac characteristic of the organ which has developed in its normal situation. The early or primary sexual differentiation of the graft gonad is apparently dependent upon the sex of the donor rather than that of the host.¹ This has already been briefly reported (Anat. Rec., vol. 37, p. 165) and will be treated at greater length in a separate publication.

In a number of grafts the gonad differs more radically from the normal ovary or testis. It consists largely of a loose, mucous type of connective tissue through which ramify occasional small blood vessels. The germ cells are found either along the periphery of the organ as an interrupted surface layer (fig. 11), or deeper, scattered in the loose connective tissue, particularly if the gonad inclines to the male type (fig. 14). It is often difficult to classify such gonads as to sex. If large growing oocytes are present, it may of course be assumed that the structure is ovarian. Since in *Amblystoma jeffersonianum* males of sixty to seventy days may sometimes possess spermatocytes in prophase (up to pachytene stages at least), the mere presence in the graft gonad of germ cells in heterotypic prophase would not prove the structure to be ovary.

The atypical gonads above described probably owe their peculiar structure to unfavorable environmental conditions. As a rule, their connection to other structures is by a very slender stalk, often of considerable length, in which, in some cases at least, no patent blood vessels can be demonstrated. Their germ cells are few in proportion to the size of the organ and, as a rule, none is found in mitosis. On reaching a certain stage of development, these germ cells apparently undergo degeneration. In a gonad of ovarian type, for example, most of the oocytes die off before reaching the diplo-

¹ That is to say, there is no complete reversal of sex previous to morphological differentiation in graft gonads developing in hosts of different sex from the donor. Such a complete early reversal appears to occur when embryos of unlike sex are joined in parabiosis (Burns, '25). Studies now in progress indicate, however, that the gonad of a graft may undergo extensive modification subsequent to its primary sex differentiation, if allowed to remain implanted for a long period in a host of the opposite sex.

tene stage; in consequence, larger oocytes are only infrequently encountered. On the whole, it would appear probable that these gonads are undergoing a slow degeneration, brought about possibly by reduced nutritive supply or by pressure from neighboring viscera of the host.

Occasionally, there are encountered small, ridge-like structures which consist only of sex-cord material and associated small cells of peritoneal origin, germ cells being entirely lacking. These correspond somewhat in structure to the sterile genital ridges which sometimes develop after extirpation of the primordial germ cells (Humphrey, '27). They are of such infrequent occurrence that it would appear highly improbable that they represent developmental stages of gonads of the types previously described. Moreover, none has been found showing indifferent cells transforming into germ cells, though such a transformation might possibly have occurred in older stages.

Of the forty older transplants which possess a distinct gonad, twenty-five have additional germ cells located outside that structure. These are almost invariably in the mesonephros of the graft, usually not far from its surface and sometimes even within its covering mesothelium. Rarely, a germ cell may be included in the epithelium of a tubule or nephrostome, as in figures 23 and 24. In the two cases in which the germ cells are not in the mesonephros they lie close to the attachment of the gonad, in one case in the body wall, in the other on the connective-tissue strand running to the gut.

The number of scattered germ cells found in these twenty-five grafts varies from one up to fifteen, except in one case in which sixty are present. In this animal a large mass of germ cells projects freely into a cavity in the mesonephros—a space apparently representing an isolated portion of the coelom. Protected here from visceral pressure and enjoying a favorable blood supply from the capillaries of the mesonephros, a group of germ cells had evidently undergone some proliferation to form a structure similar in character to a

nodule of testis. Ordinarily, however, when a small group of primordial germ cells (or a single cell) has been left in the mesonephros, surrounded completely by the stroma of that organ or included in its covering peritoneum, no cell proliferation occurs. This is evidenced by the fact that many such cells retain features characteristic of primordial germ cells long after the germ cells located in a distinct gonad have begun mitosis; note, for example, the pigment granules in the germ cell of figure 22. Since occasionally these scattered germ cells are of reduced size or atypical staining reaction, it is quite probable that they eventually undergo degeneration as do ectopic germ cells in unoperated animals.

In many grafts, of course, a distinct gonad is lacking; in these, germ cells may, nevertheless, have been differentiated. In the thirty-four grafts of fifty to seventy days' development which show no distinct gonad, fifteen show from one to eighteen germ cells, usually in the mesonephros, but in four cases located elsewhere (in peritoneum of body wall or on strand to gut). These scattered germ cells give no indication of mitotic activity, and would probably have undergone degeneration had the graft been allowed to develop longer.

Of the seventy-four grafts, nineteen (more than one-fourth) show neither a gonad-like structure nor scattered germ cells at any point. Since in grafts fixed after from ten to fifty days' growth germ cells are lacking in a smaller proportion of cases (four out of thirty-two, or one-eighth), it is quite probable that in some of these older grafts the small number of germ cells originally present had already undergone complete degeneration. In a few grafts, however, it is doubtful whether germ cells had ever been differentiated, the correlated absence of such graft derivatives as mesonephros and fat body suggesting an early sloughing or absorption of the bulk of the transplant.

CORRELATION OF GONAD DEVELOPMENT IN GRAFTS WITH STAGE OF DONOR

If the seventy-four grafts recovered fifty days or more after implantation are grouped according to the stage of the donor at operation, a significant correlation at once becomes apparent. Grafts furnished by older donors (stages 27 to 33) give rise to gonads in a much higher percentage of cases than do grafts from younger (stages 21 to 25). Of the forty grafts possessing a gonad, thirty-two, or 80 per cent, were taken from donors older than stage 25, the average age of these donors at removal of the transplant being stage 28.3. Of the thirty-four grafts lacking a gonad, only seven, or about 20 per cent, had been furnished by donors older than stage 25, the average age of these donors at the time of operation being stage 25.6.

In the above figures all the transplants removed after fifty days' growth are included, regardless of the species used. It is, however, instructive to note the results for each species and for the heteroplastic transplants from *A. jeffersonianum* donors to *A. maculatum* hosts. These are recorded in table 1.

In the homoplastic transplantations summarized in sections I and II of the table, it was customary when selecting donor and host, to use embryos from the same egg complement, selecting two of as nearly identical development as possible. It will be noted that in the case of *Amblystoma maculatum* all except one of the grafts were furnished by donors of stage 25 or younger, and the proportion developing gonads is small (one-third). The *A. jeffersonianum* grafts were obtained chiefly from donors of more advanced stages, and the proportion developing gonads is much higher (four-fifths).²

In the majority of the heteroplastic transplantations, hosts were selected four stages or more older than the donors, or vice versa. In section IIIc of table 1 it is shown that of ten

² In a series of homoplastic implantations in *Amblystoma tigrinum* in 1928 a gonad developed in sixteen out of nineteen possible cases. The donors in this series were all of stage 27 or older (average 29.9) at the time of removal of the transplant.

transplants from older donors, eight developed gonads. This is essentially the same result as was obtained in homoplastic transplants from embryos of similar stages (see section IIb of table 1). Nor does the disparity between the ages of donor and host appear responsible for the absence of a gonad in so many heteroplastic transplants from young donors into

TABLE 1
Grafts developing fifty to seventy days

SPECIES, STAGE AT OPERATION, ETC.	TOTAL NUMBER OF GRAFTS	GRAFTS WITH GONADS	GRAFTS WITH NO GONAD, BUT HAVING GERM CELLS (SCATTERED)	GRAFTS WITH NO GERM CELLS
I. <i>A. maculatum</i>				
a) Donor stage 25 or younger, host same stage	17	5	1	11
b) Donor above stage 25, host same stage	1	1	0	0
II. <i>A. jeffersonianum</i>				
a) Donor stage 25 or younger, host same stage	3	2	1	0
b) Donor above stage 25, host same stage	27	22	3	2
III. Heteroplastic donor <i>A. jeffersonianum</i> , host <i>A. maculatum</i>				
a) Donors stage 24 to 25, hosts stage 29 to 33	10	1	6	3
b) Donors stage 24 to 26, hosts stage 24 to 26	6	1	4	1
c) Donors stage 29 to 33, hosts stage 22 to 25	10	8	0	2

older hosts (table 1, section IIIa), since the occurrence of a gonad is apparently no more frequent when host and donor are of the same stage (table 1, section IIIb). The results obtained through heteroplastic transplantation merely indicate (as do the results of the homoplastic) that transplants from donors of stage 25 or younger possess markedly less capacity for the formation of a gonad than do transplants

from slightly older stages. The age of the donor, and not the age or species of the host, is apparently the important factor in determining whether or not a gonad shall develop from a given transplant.³

Hoadley ('26 b), from his studies on the potencies of chick blastoderms, concludes that isolation of a portion of the blastoderm as a graft "inhibits further differential dichotomy of the preprimordial segregates" without hindering progressive histogenesis; as a result, "the tissues and organs which develop in the grafts present a measure of the amount of organization attained by the elements of the blastoderm prior to the isolation." If one assume a 'differential dichotomy' in the intermediate mesoderm of *Amblystoma* leading to the segregation of the preprimordium of the gonad (the primordial germ cells together, probably, with closely related mesoderm), it might be supposed that in grafts from younger donors this process had been checked by transplantation before the preprimordium had become established, the gonad in consequence not developing. It has already been pointed out, however, that a gonad may be present in a small percentage of grafts furnished by younger donors (stages 21 to 25), while primordial germ cells in varying numbers may occur scattered through many such grafts in which no definite gonad has been formed. If transplants from donors of stages 21 to 25 are examined after a short period of growth in the host (ten to forty days), it is found that primordial germ cells are present in a much higher percentage of cases than are gonads in grafts allowed to develop for a longer period. Of a total of twenty-two such grafts examined, only three showed complete absence of germ cells. In thirteen

³ Assuming, of course, that the graft is implanted in a favorable situation. In a series of grafts in *Amblystoma maculatum* in 1928 many of those supplied by older donors were found to have undergone more or less complete absorption. From those remnants occasionally found, such grafts were judged to be those which had been shifted to a midventral position at the point where the anterior abdominal vein enters the liver. Few grafts actually in this position gave rise to derivatives of any size; grafts located more laterally and caudally gave rise to mesonephros, fat body, or gonad in a high percentage of cases.

of these grafts in which a satisfactory count or close estimate could be made, the number of germ cells varied from fourteen to ninety, averaging about forty-five to the graft. It is quite apparent, therefore, that the organization of the mesoderm is sufficiently advanced at the time of its transplantation to ensure the differentiation of numerous primordial germ cells in a high percentage of cases. Why, therefore, is a gonad present in so few of the grafts allowed to develop for fifty days or longer?

The answer to the above question is probably to be found in the distribution and relations of the primordial germ cells of the graft during the period shortly following its implantation. In grafts from donors of stage 26 or older, fixed ten to thirty days after implantation, the germ cells are almost invariably found to be aggregated into a group or mass projecting into the coelom (figs. 15 to 17). Eventually, these masses become relatively constricted at their point of attachment, thus giving rise to the pyriform or disc-shaped gonads already described.

In grafts from donors of stage 25 or younger the germ cells are much less frequently aggregated into a mass projecting into the coelom. In two-thirds of the grafts fixed within thirty days after implantation, the germ cells are either grouped or scattered beneath the peritoneum. Sometimes, they are arranged in a sheet or layer close beneath the mesothelium; in other grafts they are more irregularly distributed in the mesonephros or adjacent body wall (figs. 18 to 20).

The reduced numbers of germ cells found in like situations in older grafts lacking a gonad indicate the failure of proliferation of those cells which do not early acquire position in a mass projecting into the coelom;⁴ such cells may persist for a considerable period, but judging from the comparison of older with younger grafts, they eventually disappear more or less completely.

⁴ Among grafts implanted in 1928 is one in which a gonad of considerable size (a testis) is found embedded in the body wall; presumably this arose from a group of germ cells similar in their early position to those of figure 20.

The small number of gonads in grafts from young donors is therefore to be ascribed to the manner in which the germ cells have been distributed during the early growth of the graft. It cannot be attributed to absence of primordial germ cells, since these are commonly present in considerable numbers. Nor can it be attributed to the absence of any other essential component of the gonad, since a gonad is actually developed in about one-fifth of these grafts (table 1).

A comparison of transplants from older and younger donors may perhaps suggest the factors responsible for the scattering of germ cells during the early growth of those of the latter group.

In transplants furnished by older donors the cells of both ectoderm and mesoderm have undergone more division than in transplants from younger embryos; these layers, in consequence, have become relatively thin and are able to offer but little resistance to growth pressure from host tissues. The mesodermal cells, in particular, are unable to maintain their usual sheet-like arrangement. As a result, the germ cells are not held in their original position (i.e., in an elongated strand or cord), but are crowded together into a more limited area. They thus form a compact mass of cells of considerable size and, with the development of the coelom, this mass is forced to project into it (covered by a layer of mesothelium) somewhat after the manner of the normal genital ridge.

The germ layers of a younger embryo, when used as a transplant, probably suffer much less distortion from the growth of the host tissues. The mesodermal cells of the graft are still fairly large and rich in yolk platelets. Both ectoderm and mesoderm exhibit a greater firmness of consistency than in grafts from older embryos. This is noticeable in handling the transplant at operation: it shows much less tendency to curl than do transplants from donors of more advanced age, and when once implanted, it tends to remain flat and to cover a greater surface area. The larger, yolk-rich cells making up the entire mesodermal portion of the

transplant help it to withstand more effectively the early growth pressure of the host's germ layers; these mesodermal cells, moreover, probably soon resume division and exert a counteracting pressure that tends to extend the graft over a greater area. These growth interactions will determine the position of the primordial germ cells of the graft. These cells will tend to remain as a cord or strand of considerable extent (i.e., retain approximately the position they occupied at implantation) or will become even more scattered as a result of the proliferation of associated mesodermal elements in the graft. When the coelom appears, the germ cells will, as a rule, not be forced into it as a projecting mass or ridge, and a gonad will not develop.

The low percentage of gonads in grafts furnished by younger donors appears explainable, therefore, in terms of unfavorable growth interactions during the period immediately following implantation. There is no apparent absence of a necessary organization in the graft at the time it is implanted. It gives rise to primordial germ cells in a high percentage of cases and these develop into a gonad in about one case out of five. In the majority of cases, however, the germ cells are not favorably aggregated, but remain more widely distributed and in situations not conducive to further development.

In certain of the transplants furnished by older donors but nevertheless lacking a gonad, it is possible that factors other than the early growth changes in the graft may be at fault. Necrosis of a portion of the graft may, of course, eliminate many or all of the germ cells. It is possible, too, that a graft in which a gonad actually developed may be found at autopsy to show no trace of this structure. If, for example, the pressure of abdominal viscera were exerted in such fashion as to attenuate or actually rupture the attachment of the gonad to the transplant, the gonad might either undergo degeneration and absorption, or be set free in the coelomic cavity before autopsy of the host. A number of gonads recovered from grafts implanted for fifty days or

more are suspended by extremely slender stalks, suggesting the possibility of detachment as mentioned above.

That the absence of a gonad is in any case due to failure to include in the graft the necessary preprimordium seems highly improbable. In all donors examined the gonad on the right side either consists of a sterile genital ridge or an ovary or testis of limited extent, or is entirely lacking. In no donor is there to be found on the right side (from which transplants were invariably taken) a gonad equal to or even approaching the size of the gonad on the left. We may conclude, therefore, that the preprimordium of the gonad, either wholly or in very large part, was always included in the graft at implantation.

From the evidence available it would appear that a gonad develops in a graft if a number of primordial germ cells are aggregated in a favorable situation (i.e., in a mass bordering upon or projecting into the coelomic cavity). It is not believed that any gonads found in the grafts have arisen from structures once entirely free from primordial germ cells, since such structures (sterile ridges) are of rare occurrence. Moreover, the course of development of the gonad can be readily followed in grafts fixed at intervals after implantation (figs. 15 to 17). Study of such a series of grafts, like studies on the normal embryo (compare Burns, '25; Humphrey, '25, et al.), emphasizes the importance of the primordial germ cells of the urodele in the development of the sex gland. It is not contended, however, that these cells necessarily give rise to all the germ cells of the adult, since other experimental work on *Amblystoma* indicates the possibility of germ-cell origin from other elements of the developing gonad (Humphrey, '27, p. 387).

SUMMARY AND CONCLUSIONS

1. The intermediate mesoderm of *Amblystoma* embryos of germ-layer stages (21 to 34), when transplanted into the lateral body wall of another embryo, possesses the capacity for differentiation into mesonephric tubules, fat body, and primordial germ cells.

2. Mesonephric tubules develop in over 90 per cent of the grafts. A large proportion of these grafts possess glomeruli, and exhibit areas of tubule epithelium comparable to that in a normal mesonephros. Distended or cystic tubules are frequent.

3. A fat body develops in about half of the transplants. It is extremely variable in its relations to other structures, and appears able to develop quite independently of the gonad.

4. Primordial germ cells are found in practically all grafts fixed within thirty days after implantation. They differentiate in grafts furnished by donors as young as stage 21.

5. The primordial germ cells of the graft, if they become aggregated in a distinct mass projecting into the coelom, develop into a gonad, which in older grafts is found to have undergone sexual differentiation and to be similar histologically to normal ovary or testis.

6. Primordial germ cells not aggregated in a favorable situation, but scattered or entirely surrounded by other graft tissues, do not, as a rule, proliferate to form a gonad. They decrease in numbers and probably disappear entirely in the course of time, since many grafts after fifty days show few germ cells or none at all.

7. Grafts from older donors (stages 26 to 34) appear to have their germ cells favorably aggregated in a high percentage of cases. A gonad developed in about 80 per cent of such grafts. In grafts from younger donors, germ cells, though numerous, are more often scattered; a gonad developed in only about 25 per cent of such grafts.

8. A greater resistance to the growth pressure of the host tissues is interpreted as preventing the favorable aggregation of the germ cells in a large proportion of the grafts furnished by younger donors.

9. It is not believed probable that the embryonic organization essential to gonad development was lacking at implantation, even in grafts from the youngest donors used. Primordial germ cells appear in a great majority of the grafts furnished by these donors, and a gonad develops in a certain

number of cases. Moreover, the donors always show partial or complete absence of the gonad on the side from which the transplant was removed.

10. Genital ridges or gonads without germ cells are only rarely encountered in grafts at any period after implantation.

11. The primordial germ cells in the urodele appear to play a dominant part in the early development of the gonad, even though not necessarily being the only source of the definitive germ cells.

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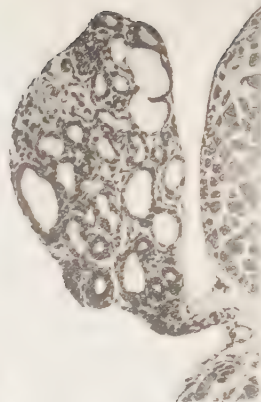
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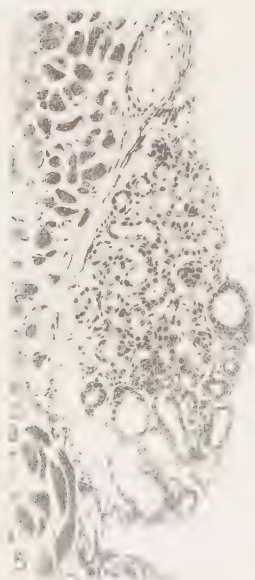
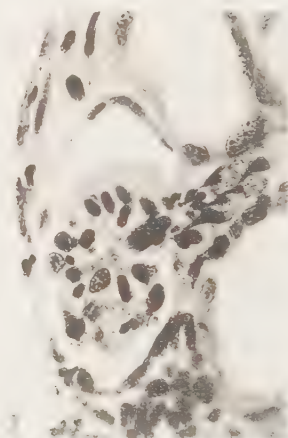
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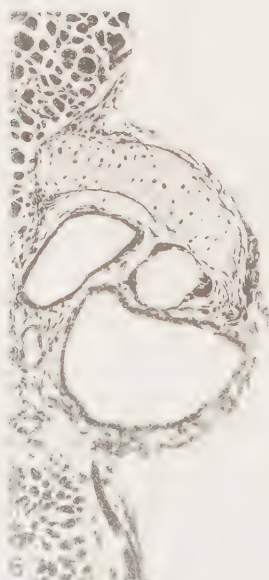
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PLATE 1

EXPLANATION OF FIGURES

2 and 3 Photographs of two host larvae (*A. jeffersonianum*), to show location and pigmentation of the graft. Both $\times 1.6$. Figure 2, host no. 274, sixty-one days after implantation of graft at stage 25, from donor of the same species and stage. Figure 3, host no. 252, sixty-three days after implantation of graft at stage 29, from donor of the same species and stage.

4 Mesonephros of stalked type, from a homoplastic transplant in *A. jeffersonianum* at stage 25, fixed after sixty-eight days. Note glomerulus near upper end and the numerous tubules which show enlargement. Magnification $\times 50$.

5 Mesonephros of broadly attached type, from homoplastic transplant in *A. jeffersonianum* at stage 29, fixed after sixty-seven days. Tubules at the upper part of the mass are of normal appearance, while those below are somewhat enlarged. A nodule of cartilage lies above the mesonephros; at its left is shown the musculature of the lateral body wall. Magnification $\times 50$.

6 Graft showing a large nodule of cartilage and greatly distended or cystic mesonephric tubules. Homoplastic transplant in *A. maculatum* at stage 25, fixed after sixty-two days. Magnification $\times 50$.

7 Glomerulus from a homoplastic transplant in *A. jeffersonianum* at stage 29, fixed after thirty-two days. Magnification $\times 240$.

8 Mesonephric tubules from a homoplastic transplant at stage 27, fixed after sixty-seven days. Magnification $\times 175$.

PLATE 2

EXPLANATION OF FIGURES

9 Ovary from heteroplastic transplant from *A. jeffersonianum* at stage 29 into *A. maculatum* host of stage 25, fixed after sixty-eight days. Both donor and host were females. Stalk attaching ovary to body wall is not included in this section. Magnification $\times 145$.

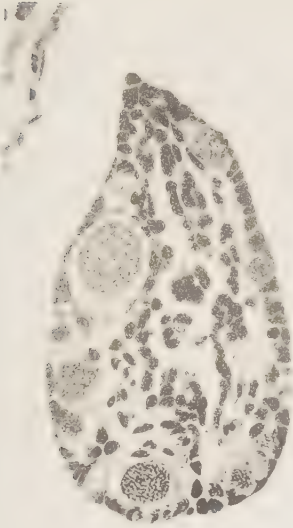
10 Ovary from homoplastic transplant in *A. jeffersonianum* at stage 29, fixed after fifty-eight days. Host a female, sex of donor unknown. This ovary, of flattened form, attaches by its edge to a similarly flattened fat body, which in turn attaches by its edge to the body wall. Magnification $\times 102$.

11 Atypical ovary from homoplastic transplant in *A. jeffersonianum* at stage 33, fixed after sixty-four days. Both donor and host were females. Note excess of mucous connective tissue. With its peripheral germ cells (some growing oocytes) and its central cavity, this gonad is decidedly ovarian in structure. Magnification $\times 145$.

12 Testis from heteroplastic transplant from *A. jeffersonianum* at stage 29 into *A. maculatum* host of stage 25, fixed after sixty-one days. Both donor and host were males. Fat body of graft lies above gonad, and a part of a large mesonephric cyst is shown below it. Magnification $\times 145$.

13 Testis from a homoplastic transplant in *A. jeffersonianum* at stage 27, fixed after sixty-seven days. Both donor and host were males. This gonad developed as an enlargement on the strand connecting graft and intestine; as this was sectioned transversely, the connection of the gonad to other structures is not apparent in any section. Note the darkly stained cords of cells (the 'sex cords' or anlagen of the duct system) and the scattered germ cells, features characteristic of the testis. Magnification $\times 145$.

14 Atypical testis from a homoplastic transplant in *A. jeffersonianum* at stage 33, fixed after sixty-four days. Donor male, host female; a similar atypical testis may be found, however, when both donor and host are males. Compare atypical ovary of figure 11 with atypical testis; note in the latter the absence of a central cavity and the scattering of germ cells, but few of which are peripheral. Magnification $\times 145$.



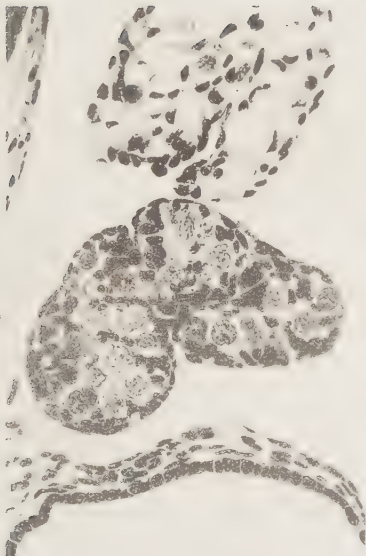
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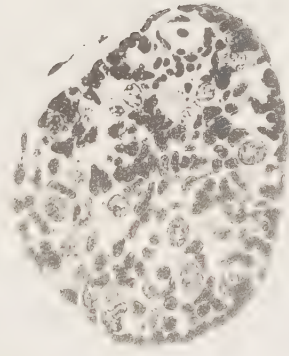
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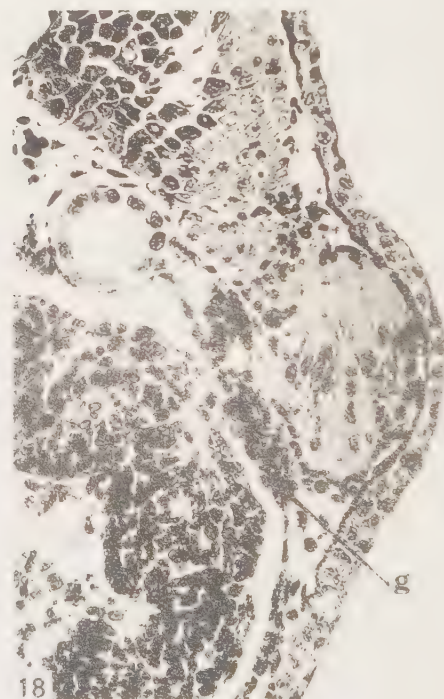
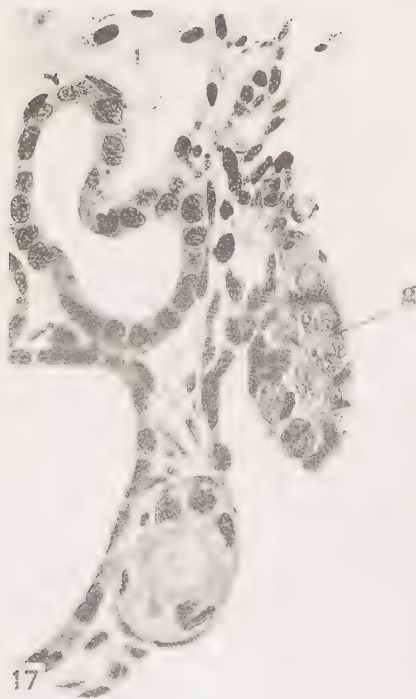
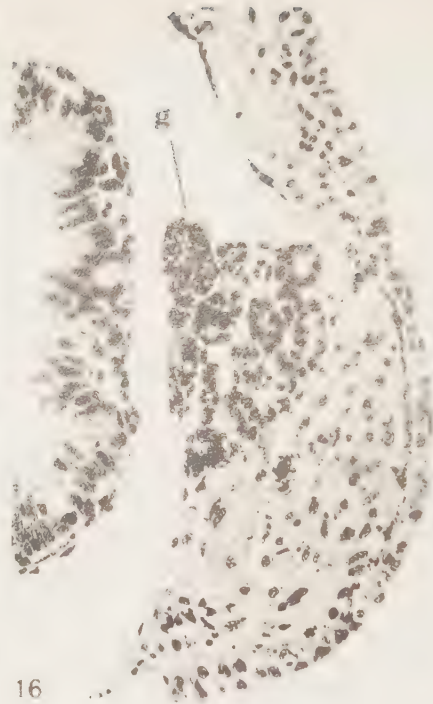
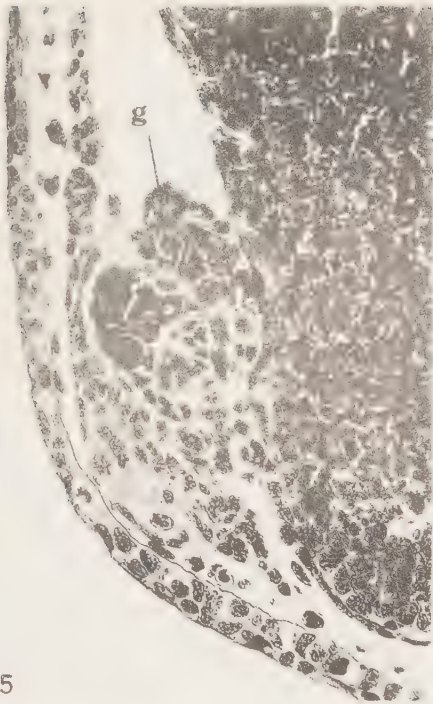


PLATE 3

EXPLANATION OF FIGURES

Figures 15 to 17 illustrate three stages in the development of a gonad in transplants.

15 Homoplastic transplant in *A. jeffersonianum* at stage 29, fixed after eleven days. Only the ventrolateral portion of the body is shown. The primordial germ cells (*g*) form a mass bulging into the coelom. At the right are entodermal cells heavily laden with yolk platelets; at the left are the ectoderm and somatic mesoderm of the body wall; below the germ cells is a group of developing mesonephric tubules (darkly stained). Magnification $\times 156$.

16 Homoplastic transplant in *A. jeffersonianum* at stage 27, fixed after eighteen days. Only the ventrolateral portion of the body is shown. The primordial germ cells (*g*) have now lost their yolk material, but contain considerable pigment; they form a distinct mass extending into the coelom from the mesonephros of the graft. Mesonephric tubules are now apparent; below these, and making up a large part of the bulk of the graft, are skeletal-muscle fibers derived from the implanted axial mesoderm. At the left is a part of the intestine of the host. Magnification $\times 156$.

17 Homoplastic transplant in *A. jeffersonianum* at stage 29, fixed after thirty-two days. A part of the mesonephros of the graft is shown, with the gonad attached to its surface. The primordial germ cells still contain pigment masses in their cytoplasm; the cell marked (*g*) is in a telophase stage of mitosis. Magnification $\times 240$.

18 Transplant showing primordial germ cells (*g*) not aggregated into a mass projecting into the coelom. Compare with figure 15. This graft lies unusually far dorsad (note its position just lateral to the wolffian duct of the host) and is connected to the gut by a short, thick strand of mesodermal cells. Homoplastic transplant in *A. jeffersonianum* at stage 24, fixed after twelve days. Magnification $\times 156$.

PLATE 4

EXPLANATION OF FIGURES

Figures 19 and 20 show grafts more advanced in development than that of figure 18, but with the germ cells (*g*) in each case not aggregated into a gonad-like mass projecting into the coelom. In figure 19 the germ cells lie in the edge of the graft mesonephros; in figure 20 they are, except for one cell, just beneath the peritoneal epithelium. Both figures are of homoplastic transplants in *A. maculatum*; that of figure 19 was implanted at stage 23 and fixed after twenty-six days, while that of figure 20 was implanted at stage 25 and fixed after twenty-three days. Magnification $\times 156$.

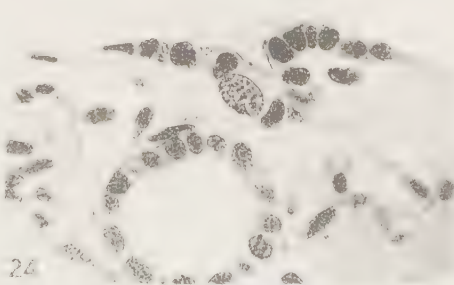
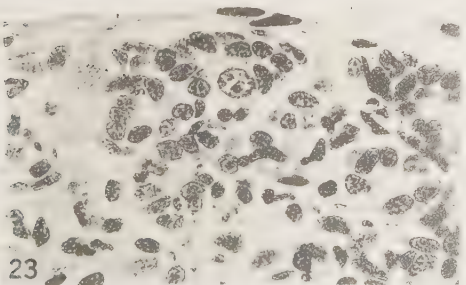
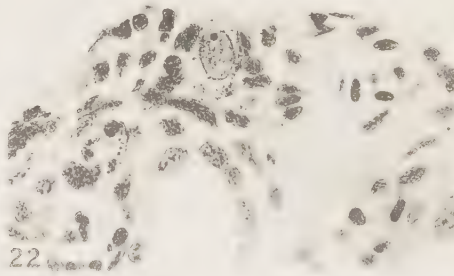
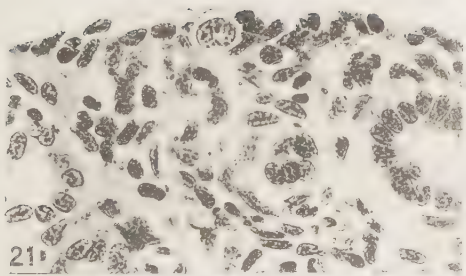
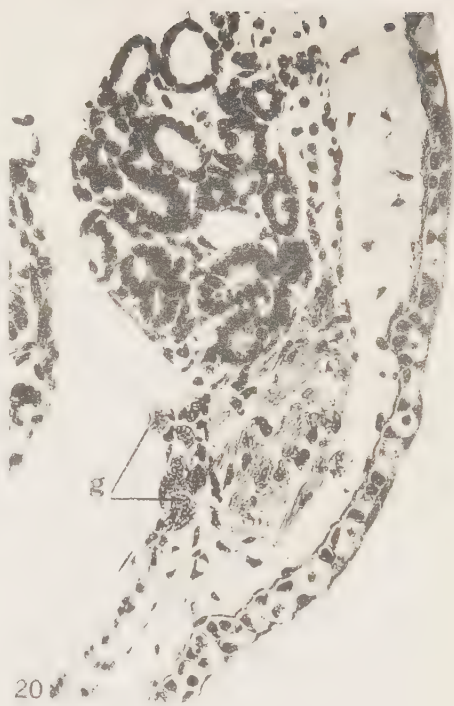
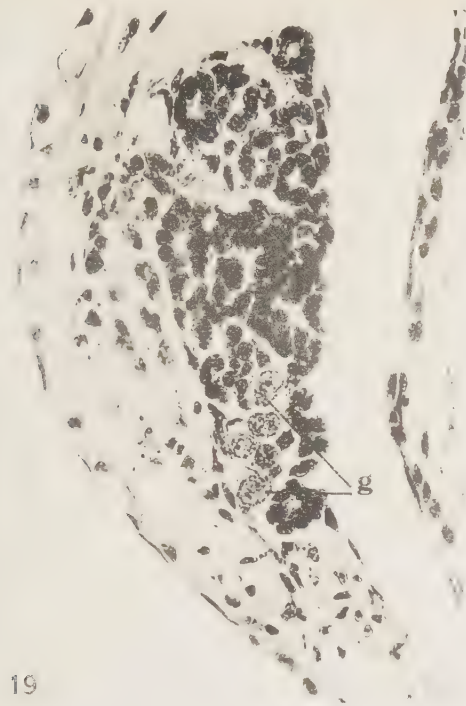
Figures 21 to 24 show isolated primordial germ cells in grafts more advanced in development than those of figures 19 and 20.

21 Germ cell with irregular nucleus, lying in the peritoneum over mesonephros. Homoplastic transplant in *A. jeffersonianum* at stage 25, fixed after sixty-one days. Magnification $\times 240$.

22 Germ cell with numerous pigment granules, in peritoneum over mesonephros. Homoplastic transplant in *A. jeffersonianum* at stage 30, fixed after fifty-five days. Magnification $\times 240$.

23 Germ cell in epithelium of a mesonephric tubule. Heteroplastic transplant; host *A. maculatum* of stage 25, donor *A. jeffersonianum* of stage 29. Graft fixed sixty-one days after implantation. Magnification $\times 240$.

24 Germ cell in epithelium of nephrostome, from same transplant as that of figure 22. Magnification $\times 240$.



THE METHODS FOR THE DEMONSTRATION OF THE
GOLGI APPARATUS

V. THE IDIOSOMIC COMPONENT. METHODS FOR LIPOIDS.
TROPHOSPONGIUM, LACUNOME, AND CHROMIDIA

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THE IDIOSOMIC COMPONENT OF THE GOLGI COMPLEX

The preceding papers in this series (Bowen, '28 a, b, c, d) have dealt mainly with those methods which demonstrate what may be provisionally referred to as the Golgi material proper. But, as is well known, in some cases this material is intimately associated with another substance which is in many respects its technical opposite. Thus, while the Golgi material is intensely osmiophilic, is often stained by Fe-

hematoxylin, and reacts positively to silver, this accompanying substance is very chromophobic and in the highest degree refractory toward all known methods of technic. This second component has been variously named, the one which I have usually used being the 'idiosomic' material. The Golgi material proper and the idiosomic material together constitute a duplex structure—the Golgi complex (see, for example, Bowen, '26 b). This idiosomic substance is best known in male germ cells where it has long been familiar to cytologists under various titles. There may still be some slight question as to the reality of this material and its relation to the Golgi apparatus proper, but in the spermatocytes and spermatids of many animals the appearance certainly is that of a tangible material which has a very close association with the chromophilic Golgi substance. The attempt to extend this conception of the duplex constitution of the Golgi apparatus to all kinds of cells has not yet resulted in a satisfactory conclusion (Bowen, '26 a), and the whole matter is in much too uncertain a phase to warrant any decided statement either one way or the other. If the presence of this chromophobic material could be proved in all cells, our understanding of the whole Golgi apparatus problem would be at once greatly advanced.¹

Without, therefore, attempting any definite estimate of the validity of the foregoing suggestions, it is here the place only to inquire into the possibility of demonstrating such an idiosomic material in any cells by means of positive, and to some degree specific, stains. This query brings a largely negative answer, and in this place it is possible only to review several suggestive methods without thereby implying that they necessarily have any widespread application or significance. Most of these methods seem applicable only to the male germ cells, but it would be of much interest and importance to know whether they might have a wider meaning. Much remains to be done by someone who will successfully inquire into the technic of demonstrating the idiosomic material wherever it exists.

¹ But see also Hirschler's ('27) very recent discussion of this problem.

Method of Prenant (Prenant, '02, '05; also Langeron, '25, p. 511)

This method has been claimed by some French authors to be applicable to the idiosomic substance. It has apparently been applied with success by Champy ('23) to this material in the male germ cells of *Discoglossus*, but the possible extent of its usefulness seems to me very problematical.

1. Fix in Bouin or Perenyi (Flemming is not so good).

2. Cut sections 3 to 5 μ and stain as usual with Fe-hematoxylin.

3. Then stain strongly in methyl-eosin or erythrosin, the latter being preferable. Langeron ('25) states that methyl-eosin is only soluble in alcohol, erythrosin in water; make up the solutions (of 1 per cent) accordingly and stain for about ten minutes. Langeron, and also Prenant, in a later paper ('05), reverse the order of staining and apply the eosin dye first.

4. Wash carefully in water and treat with a strong aqueous-alcoholic (Langeron uses a 1 per cent solution in 90 per cent alcohol) solution of light green for a few (two to three) seconds. This is the most difficult point, and if the green acts too long, the red dye is removed and a very poor preparation results.

5. Rinse in alcohol (not water), dehydrate, and mount as usual. The idiosomic material should be colored green.

Needless to add, this method was not originally developed for the purpose here suggested—but for connective tissue, and the detection of mucin which, it is interesting to note, is of proteid nature. This method has also been applied by Poisson ('27) to the spermatids of *Notonecta* (after Bouin fixation) with interesting results.

Methods of Papanicolaou and Stockard

These authors (Papanicolaou and Stockard, '18) have devised four variations of a fuchsin-methylene blue-staining combination, with which they achieved some very striking results on the idiosome in the guinea-pig's spermatocytes and early spermatids. The methods have not been tried on other material, though they suggest possibilities of wider application, at least to vertebrate germ cells.

1. Fix in Zenker for eighteen to twenty-four hours.
2. Embed in paraffin, and cut sections at 3 to 5 μ .
3. Stain according to the following four variations, which give various effects on the idiosome and the acrosomal material—completely described in the original paper:

Method A. Bring sections down to water and cover for a few seconds with Lugol's solution, and then wash in water until the yellow color begins to fade out. Place in a saturated aqueous solution of acid fuchsin for one-half to one minute—then run up to carbol-xylol and mount.

Method B. Same as method A, but after staining with acid fuchsin bring the sections into 80 per cent alcohol and then for a few seconds, ten to twenty, in a saturated solution of methylene blue in 80 per cent alcohol. Then proceed as before.

Method C. Pass the slides down to 80 per cent alcohol and then stain for several minutes in the methylene-blue solution as above. Then pass down to water through the usual alcohols and leave for a few minutes in a 2 per cent Fe-alum solution until a pronounced bluish tone is obtained. Wash thoroughly in running water until the blue tone begins to disappear and cover for a few seconds with Lugol's solution. After this, wash again in running water (a few minutes) until the yellow color begins to fade. Then stain in acid fuchsin for from one to two minutes, pass up through the alcohols to carbol-xylol and mount.

Method D. Same as method C, with the difference that, after the fuchsin stain, the slides are brought into 80 per cent alcohol and then to the methylene-blue solution, given above, for a few seconds, thence to carbol-xylol and mount.

With method A the idiosomic material is rose color, with method C it has a pronounced violet color. The authors state that a good Zenker fixation is a prime requisite, and in its absence a dark, diffuse stain results with loss of nice differentiation.

In addition to the foregoing methods which seem to have a special application to the male germ cells, it should also be noted that many of the customary staining methods also yield results, usually of a diffuse nature, on the idiosome in male germ cells. Unfortunately, these methods, as well as the more special ones noted above, fail to yield decisive results in cases where the idiosomic material is not in a concentrated condition. Thus all these methods break down in just those settings where they are most wanted for critical studies (e.g., dividing spermatocytes and resting somatic cells).

Weigl's fuchselin method (Weigl, '10 a)

One further method remains for consideration—in many respects more promising than the preceding ones. Unfortunately, this method has been little studied by workers other than Weigl, at least from the point of view he suggested, and it is impossible as yet to gauge its real significance. Weigl ('10 a) stained Kopsch preparations (see part III, p. 245) with fuchselin (see section in this paper on the trophospongium, p. 118) and observed that in cells blackened with osmic acid the fuchselin apparently did not stain the Golgi apparatus, while in cases of incomplete blackening (or after the blackening had been extracted by bleaching) something corresponding in position to the Golgi network could be stained with fuchselin (in vertebrate somatic cells). From this he concluded that the Golgi apparatus had a duplex structure,² one constituent blackening with osmic acid (the myelinogenous or Golgi material proper), the other staining positively with fuchselin. Or, as Nussbaum ('13), in whose laboratory Weigl worked, later reviewed the problem, the Golgi apparatus consists in all cases of a lecithin-like constituent which is blackened by osmic acid, and another substance not blackened by osmium, but staining intensely with fuchselin, the proportions of these two components being different in different conditions of the apparatus as a whole. In a later paper Weigl ('10 b) states that, after many fixatives which destroy the osmiophilic component, it is still possible to stain the other material with fuchselin, and with Fe-hematoxylin or Benda's crystal-violet method (in the nerve cells of cephalopods).

If this interpretation given by Weigl be correct, many puzzles raised by the studies of Holmgren on his trophospongium would be automatically cleared up and at the same time the vexatious problem of the structure of the Golgi apparatus would be resolved. Certainly, it would be well worth while to reexamine the whole matter from the point

² This is, I think, the original statement of this view.

of view suggested by Weigl's work and with the use of his methods of attack. Thus far only Kopsch ('26) has ventured any opinions in this direction, and, from an extended study with different technical methods, he arrives at a conclusion in essential harmony with that of Weigl. According to Kopsch, the Golgi apparatus consists of two different substances, one osmiophilic (O-substance) and constituting what I have referred to as the Golgi material proper, and the other (T-substance) forming the so-called trophospongium (of Holmgren). In different kinds of cells these constituents have different proportions, thus explaining the differences in embryos, adults, different animal species, etc., of the impregnation, preservation, and staining of the Golgi apparatus. Kopsch concludes that the O-substance is not preserved in acid fixing fluids, while the T-substance is preserved in trichlorolactic and trichloroacetic acids as well as in all fluids containing chromic acid, or chromic-acid salts, together with osmic acid. This work is certainly suggestive of the interesting possibilities to be finally tested out by further work along the same lines.

METHODS FOR THE DEMONSTRATION OF LIPOIDS AND FATS

The question as to the chemical nature of the Golgi material is still an unsettled one; indeed, diametrically opposite opinions are held by different schools of workers. The old view, based largely on the behavior of osmic acid,³ has been that the Golgi material is essentially of fat-like affinities (myelin, lecithin-like, etc.). This seems to have originated with v. Bergen ('04), and has been accepted in its essentials by Sjövall ('06), Weigl ('10 a), Poluszyński ('11), Mulon ('12), Cajal ('14), Hirschler ('14), and numerous more recent workers. On the other hand, Parat and Bergeot ('25) assert that lipoidal materials have nothing whatever to do

³ It is, of course, clearly recognized now that blackening with osmic acid is not necessarily a proof of the presence of lipoidal material. But it should also be borne in mind that by no means all fatty substances give a positive reaction with osmic acid (e.g., see brief discussion in Kingsbury, '11).

with the Golgi apparatus (their vacuome). Kopsch ('26) seems also inclined against a lipoidal interpretation of the apparatus, but leaves the matter undecided. As between such extraordinarily divergent opinions it is at present quite impossible to arrive at any decision regarding the chemical nature of the Golgi apparatus. Nevertheless, the fact remains that if the Golgi material is a fat-like substance it should be possible to demonstrate it by suitable microchemical tests. Unfortunately, the attempt to apply such tests yields equivocal results, especially in view of our extensive ignorance regarding tests which are applicable with certainty to various kinds of cellular lipoids. With present methods, failure to secure a positive result can, therefore, hardly be accepted as final. The situation is just now decidedly confusing.

Positive results on the Golgi material have, however, been reported by a few workers, and these will be reviewed with the hope that further efforts in this direction will clarify the situation.

Ciaccio's methods (Ciaccio, '10)

1. Fix for twenty-four to forty-eight hours in the liquid of Ciaccio:

5 per cent Potassium bichromate,	100 cc.
Formalin,	20 cc.
Formic acid, C.P.,	10 to 15 drops
or Acetic acid,	5 cc.

Material preserved in formalin is treated like fresh material. Good results are also obtainable with fixation in Zenker, Foà, Helly, and Bouin.

2. Transfer the pieces to 3 per cent potassium bichromate for a little over one week at room temperature. During the hot months, or in an incubator at 37°, six to seven days are sufficient.

3. Wash in running water for twelve hours; then 70 per cent alcohol for twelve hours, 95 per cent alcohol for six hours, and one or two hours in absolute. Then embed in paraffin by way of xylol, chloroform, or preferably carbon bisulphide.

4. Sections are affixed to the slide by the method of Henneguy, brought down to 70 per cent alcohol and then stained for thirty to forty-five minutes, and preferably at 25° to 30°, in a saturated

solution of sudan III in 80 to 85 per cent alcohol. Scharlach R, indophenol, and Nile blue may also be used, but are less favorable. In any case, the staining solution should be kept in the incubator, cooled at the moment of using and filtered.

5. Decolorize in 50 per cent alcohol, wash freely in distilled water and counterstain with:

a) haemalum or hematein with or without a weak stain in light green; or *b)* water blue or crystal violet; or *c)* Fe-hematoxylin.

6. Mount, after washing in distilled water, in Apáthy's gum-and-syrup medium.

Ciaccio gives also two variations involving treatment with a bichromate-osmic mixture, or fixation in such a fluid. These methods are similar to the postchroming processes described in part IV, page 371. Further slight variations in two of his methods are also given by Ciaccio, but the directions as given above are the most useful.

Ciaccio ('10) gives figures of the testis of the mouse in which the area of the Golgi apparatus is very sharply stained (especially his fig. 11) (with sudan III). Karpova ('25) reports positive results with sudan III in the male germ cells of *Helix* with frozen sections, but no result with sections cut in paraffin. Parat and Painlevé ('26) report positive results on similar material. Weiner's ('26) results are the most clean-cut of all—on the intestinal epithelium of the mouse. His method is a slight modification of Ciaccio's, as follows:

1. Fix, omitting the formic or acetic acids entirely.
2. Cut sections with a freezing microtome.
3. Stain in sudan III for a very long time (from two to four to seven days).

Smith's method (Smith, '08)

Sometimes called the Lorrain Smith method.

1. Fix material in formalin.
2. Cut sections with the freezing microtome and stain in a watery solution of Nile-blue sulphate.

Karpova ('25) on male germ cells of *Helix* finds the Golgi rodlets stained blue by this method. (See also, in connection with this method, Smith, '06).

Dietrich's method (Dietrich, '10)

Usually called the method of Smith-Dietrich because it is based in part on the method of Smith and Mair ('08 and '09).

1. Fix in formalin for one to two days.
2. Cut frozen sections.
3. Place in saturated aqueous solution of potassium bichromate at 37° to 40°C.
4. After twenty-four, and again after forty-eight hours, wash samples in water and place in Kultschitzky's⁴ acetic acid-hematoxylin for four to five hours at 37° to 40°C.
5. After washing overnight in water, differentiate in Weigert's borax-ferricyanide of potassium.
6. Finally, wash thoroughly in water and mount in levulose.

Sections may be mounted in balsam, but some stained lipoids are not alcohol and xylol fast. Counterstaining in safranin is possible, but usually not practiced. The chief point is that of the temperature (step 3) which must be varied for different materials, sometimes as high as 60°, for mordanting. The second point of importance is the duration of step 3 (mordanting), neutral fats with unsaturated radicals staining only after perhaps three weeks. The technic must be continually altered according to the material—see original for specific examples. Karpova ('25), again on male germ cells of *Helix*, finds the Golgi rodlets stained black by this method; and Parat and Painlevé ('26)⁵ also report a positive stain on similar material.

⁴ Mallory and Wright give the following formula for this stain:

Hematoxylin,	1 gram
Alcohol,	10 cc.
Acetic acid,	2 cc.
Water,	to 100 cc.

⁵ These same workers also report on similar material positive results with the albuminoid tests of Millon and Derrien-Turchini, and the phosphorus tests of MacCallum and Cretin. But it should be noted further that Parat and his coworkers disagree with the general interpretation of the structures thus stained in terms of Golgi material, and prefer to regard them as of mitochondrial derivation (the lepidosomes).

Kingsbury's method (Kingsbury, '11)

This is a modification of Weigert's method.

1. Fix in Zenker for two days, reducing the acetic-acid content to 1 per cent.
2. Mordant in Müller's fluid for ten to fourteen days at 38°C.
3. Stain by Weigert's hematoxylin method of 1885, which seems preferable to the Pal-Weigert method.
4. In differentiating, dilute the potassium ferricyanide-borax bleacher from three to four times.

Judging from Kingsbury's remarks on the application of this method to mitochondria, it would be well to reduce the acetic-acid content of the fixative still further or eliminate it entirely when working with the Golgi apparatus. Cowdry ('12) reports clear, positive results by this method on the nerve cells of the pigeon.

Karpova ('25) reports negative results with Fischler's method for fatty acids. Weigl ('10 a) states that on mammalian nerve cells frozen sections of formalin material stained with sudan III or scharlach R, stained very faintly or not at all.

Ciaccio's methods for unmasking histolipoids (Ciaccio, '26 a)

The most recent work on this problem is the extensive investigation just published in preliminary form by Ciaccio (e.g., '26 a). Ciaccio looks upon the fatty substances in cells as of two kinds: 1) products of metabolism varying in accord with the functional state of the cells, and, 2) essential morphological constituents, true cellular constants, which only undergo sensible oscillations in abnormal conditions. It is this second class which is termed 'histolipoids' by Ciaccio and which include the Golgi complex (his 'formation x'). These histolipoids are said to be in a 'masked' condition because they do not respond in general to the usual microchemical tests for fat. In order to demonstrate them, they must first be 'unmasked' or 'liberated.' The exact explanation of this phenomenon in chemical terms is not yet

definitely determined. The unmasking process must be accomplished in conjunction with a fixation of the tissues which will permit of the localization and subsequent histological study of the histolipoids. The methods for accomplishing this (Ciaccio, '26 a and b) may be of two general types, as follows:

I. Methods in which the unmasking is effected *after* preliminary fixation in formol. Thus one may fix in formol six to twenty-four hours and then treat for twenty-four to forty-eight hours at 37°C. with trypsin (Grübler). Or one may fix in formol for twelve to twenty-four hours and then treat with 1 per cent phenol at 37°C. for twenty-four to forty-eight hours. Acid or alkali solutions and pepsin may also be used, but apparently the phenol method is the best. The intensity of the final result is dependent more or less upon the duration of fixation, on the action of the lipoid-liberating agent, and on the portion of a piece examined (more intense action at the periphery).

II. Methods in which the unmasking is effected at the same time as the fixation. Thus one may fix in: 1) the formol-uranium nitrate of Cajal, or the formol-cobalt nitrate of Da Fano (part II) for eight to twelve hours; 2) or fix for six to twelve hours in uranium nitrate, 1 gram; 5 per cent potassium bichromate, 85 cc.; distilled water, 15 cc.; 3) or fix for some hours in 70 to 80 per cent alcohol; or in alcohol, 60 cc.; distilled water, 20 cc.; formol, 20 cc.; with successive passage for a few minutes into a mixture of alcohol plus 5 per cent calcium chloride; 4) and many other substances, such as salts of cadmium, zinc, mercury, some metalloids, and methyl alcohol.

After preliminary treatment by either method I or II (except method 2 under II), the pieces are left a few minutes in a 2 per cent solution of cadmium chloride (CdCl_2), and thence are postchromed in 5 per cent potassium bichromate with acetic acid in the proportion of 1 to 2 per cent, for five to six days at room temperature, or for two to three days at 37°C. Then the pieces may be sectioned by freezing or embedded in paraffin after the usual treatment.

The sections may be stained: 1) with a saturated alcohol-acetone solution of sudan III for one hour, decolorized in 40 to 50 per cent alcohol; water; nuclear staining with hematoxylin, and, if one wishes, a light background stain of light green. The lipoids are colored orange or orange-red; 2) with chromic-hematoxylin according to Smith-Dietrich. The lipoids are colored blue-black. Though not as specific as sudan III, an elective coloration of the unmasked lipoids can be obtained by regulating the differentiation in the borax-

ferricyanide solution. In general, the mixtures of Cajal and Da Fano give the most favorable results.

Ciaccio ('27) has applied these methods particularly to the idiosomic complex in the male germ cells of *Discoglossus*, with positive results, for the details of which reference may be made to the original communication.

The results of these microchemical tests are certainly suggestive, if not finally conclusive. They tend to indicate, in my opinion, that further efforts will yield more complete and satisfactory results, and assuredly bear out the old views as to the lipoidal nature of at least some element in the Golgi apparatus. It should be noted that, in general, the method of frozen sections is more likely to be successful than embedding in paraffin, and that cells with abundant and well-marked Golgi material are more likely to respond successfully. Efforts in this direction should certainly be carried further, and it seems to me not unlikely that a thorough-going effort, even with the methods now known,⁶ would yield much positive information of interest. In any event, the possibility that the Golgi apparatus has lipoid affinities cannot yet be summarily dismissed, as some have tried to do.

THE TROPHOSPONGIUM

Shortly after Golgi's first publication on the 'internal reticular apparatus' appeared Holmgren's initial paper of a long series in which, over a period of fifteen years, he gradually developed his well-known trophospongium theory. After the lapse of more than ten years since the appearance of Holmgren's final paper, cytologists are still at odds over the reality and interpretation of the canal-like spaces and trophospongial networks which Holmgren described and figured in so many kinds of cells. The general opinion seems to be that the trophospongium is really the Golgi apparatus demonstrated in a somewhat unusual guise, because of the

⁶ Including, for instance, the method of Herxheimer, which has not yet been adequately reported upon in connection with the Golgi apparatus.

nature of the fixatives and stains employed. A few, however, have not agreed with this conclusion, notably Golgi himself. Another group has found both Golgi apparatus and extensions from other cells present together in the same cell, thus providing in a few cases both the classical network of Golgi and the canals of Holmgren. It is clear, however, that in at least some cells the trophospongial canals are not demonstrated in the form which the Golgi apparatus is known to possess, and this was clearly recognized by Holmgren ('14) himself, who believed that his 'canals' represented a condition of the Golgi network which osmic or silver preparations were not capable of demonstrating. The connection between the network and peripheral 'trophocytes,' invariably found by Holmgren, is still unexplained, since the true Golgi apparatus has been found by most workers to have no connection with the cell periphery.

In spite of these many contradictions, there seems to me to be a preponderance of evidence in favor of the homology of Golgi network and trophospongium in many cases at least. In special cases it is possible that actual extensions of one cell may penetrate into the cytosome of another cell, as Holmgren asserted. The whole matter calls for an extended reexamination making use of the same materials and methods which Holmgren employed together with the most favorable special methods for the Golgi apparatus itself. Coupled with these studies an extended examination of the whole problem of intracellular vacuoles is called for (see also part VI on the vacuome). Through such a study we may hope further to clarify our morphological conception of the cell at least.

This section will deal primarily with the methods used by Holmgren and recommended by him as most favorable for the trophospongium and 'canals.' Some of these have already been noted in part IV in the section on general methods of fixation and staining. Many of these give so-called 'negative' images, while the more specialized ones yield positive stains. From the action of these latter (fuchselin) methods

Weigl and Kopsch have concluded that the trophospongium is merely a particular part of the material of the Golgi apparatus, as noted in the preceding section. The methods employed by Holmgren may be divided into two classes according to their effect on the trophospongium. According to Holmgren (e.g., '02 c), the trophospongium represents 'solid' ingrowths into a cell of processes from an external cell or trophocyte. Under certain metabolic conditions, these trophospongial networks, according to Holmgren, become liquefied and are thus transformed into large spaces, the 'canals' (or 'Kanälchen,' previously called 'Saftkanälchen'). Since these canals appear only under certain circumstances dependent on conditions in the individual cell, they are not always demonstrable. When they do appear, they are seen as conspicuous, clear, unstained spaces set off by contrast with the darker, positively stained cytoplasm. Such results follow the use of a great variety of the common fixatives (most of those listed below under the head of 'General Methods'). The true trophospongial networks—those, that is, which have not become liquefied or canalized—on the contrary, can as a rule be stained only by special methods, such as those devised by Holmgren, involving fixation in trichlorolactic (or trichloroacetic) acid and staining with Weigert's elastic fiber method (fuchselin). These latter methods yield positive stains of the trophospongial networks. These distinctions, carefully set forth by Holmgren, are often overlooked in present-day discussion of his theory and methods.

General methods employed by Holmgren

The following partial list of citations will give a very complete idea of the range of common fixatives and stains with which Holmgren obtained results on the 'Kanälchen.' Details were rarely given by Holmgren as to his use either of fixatives or stains, the presumption being that the methods followed the usual mode of their handling in histological laboratories.

Mercuric chloride (saturated solution in normal saline). Stain in carmine-indigo carmine or Delafield-acid fuchsin-orange ('99 a).⁷

Sublimate-acetic (100:5). Stain with Fe-hematoxylin combined with rubin or orange, or with toluidin-erythrosin (2 per cent toluidin for twenty-four hours, and differentiate with 0.1 per cent erythrosin) ('99 c).

Sublimate-picric (Rabl). Stain with toluidin-erythrosin as noted under 'sublimate-acetic' above ('99 b, '99 c, '00 a, '00 d, '02 d, etc.); allow the erythrosin to stain as far as possible ('00 d). Or stain with Fe-hematoxylin with or without rubin or orange ('99 c, '00 d, '02 d); or acid fuchsin-orange-Fe-hematoxylin (using Squire's formula, 1 gram acid fuchsin, 6 grams orange, in 60 cc. alcohol and 240 cc. water, staining about five minutes) ('00 c); or thiazinrot R-toluidin ('02 d, '03 a).

Sublimate-alcohol-acetic (Apáthy's 75:25:5). Fix not more than five to six hours, and then transfer immediately to 95 per cent alcohol ('00 d). Stain with toluidin-erythrosin ('99 c, '00 a, '00 d); or Fe-hematoxylin ('00 d).

Heidenhain's sublimate-trichloracetic. No details given ('14).

Zenker. Stain with Fe-hematoxylin ('02 a).

Helly. Stain with Fe-hematoxylin and eosin or erythrosin ('14).

Alcohol-chloroform-acetic (Carnoy). Stain with toluidin-erythrosin ('99 c, '00 b, '02 d); or Fe-hematoxylin alone or with rubin or orange ('99 c, '02 c, '02 d, '02 e, '03 b), or the Fe-hematoxylin-acid fuchsin-orange combination ('00 b, '03 b); or thiazinrot R-toluidin blue ('02 d, '03 a); or Weigert's elastin stain ('00 b and d).

Flemming. Stain with Fe-hematoxylin ('02 c and e).

Benda's Flemming. Stain with Fe-hematoxylin or Benda's crystal violet ('10).

Lindsay Johnson. Stain as for Benda's Flemming above ('10).

⁷ The references are all to Holmgren's papers.

Golgi's chrome-silver method. Fix for six to eight days at 30° to 31° Cels. in 4 per cent potassium bichromate four parts and 1 per cent osmic acid one part. Silver in 0.75 per cent silver nitrate for twenty-four to forty-eight hours at the same temperature. Wash out for one day in numerous changes of alcohol and embed ('08 and '10).

The preceding notes include about all that Holmgren ever published as to the details of his general procedure. One is obviously obliged to proceed largely on the basis of personal experimentation. The number and indefiniteness of Holmgren's methods constitute one great difficulty in reproducing his results with any certainty.

In addition to these general methods for the canals (the last three, however, applying more particularly to the trophospongium), Holmgren also published two special methods for the trophospongium proper, which were evolved from trials with many reagents in an endeavor to find one which would give a suitable fixation for subsequent application of Weigert's elastin stain. These methods are given below.

The trichloroacetic method (Holmgren, '02 a. See also '04 and '08)

1. Fix for eight up to at most twenty-four hours in a 2 to 5 per cent solution of trichloroacetic acid. (Apparently the usual strength for routine work was 5 per cent.)

2. Run up through (40), 50, 60, 70, 82, and 96 per cent alcohols for twenty-four hours each, and embed in paraffin as usual.

3. Cut sections from 2 μ to at most 5 μ thick and stain for twenty-four hours in Weigert's resorcin-fuchsin (fuchselin), which must be *freshly* prepared.

4. Treat with alcohol and mount as usual.

The most important matter is the preparation of the stain. Sometimes it works well and sometimes not, the reasons in either case being unknown. But it is very noteworthy that the staining mixture is characteristically usable only when just prepared. This, Holmgren thinks, may be due to the formation during the preparation of the stain of a very unstable combination in addition to the characteristic elastin stain. It is perhaps this unstable combination which (after proper fixation) possesses the elective staining properties for the trophospongium. In preparing the stain it is absolutely essential

to have the iron-chloride solution very concentrated and to use the 'resorcinum resublimatum pur.' The iron-chloride solution must (according to Holmgren) be the *Liquor ferri sesquichlorati*, Pharm. Germ. III.⁸

Proceed as follows (these notes are a combination of the directions given by Holmgren and the more complete description of the operations as originally given by Weigert, '98).

1. 2 grams of basic fuchsin and 4 grams of resorcin are dissolved in 200 cc. of distilled water.

⁸ This solution of iron chloride can only be prepared by a somewhat tedious and complicated process. I have found it very difficult to obtain a copy of the original source as given above by Holmgren, but having finally succeeded and knowing of no readily accessible place where the procedure is described, I have thought it might be of value to give here the exact details. The latest (VI) edition of the *Pharmacopœa Germanica* gives no directions for preparation, and those of the second edition and also of the U. S. P. differ not only among themselves, but also from the formula as given in the third edition quoted specifically by Holmgren. The directions in the third edition are given on pages 185 to 186 as follows (abridged):

One part of iron (pure iron wire cut into small pieces is suitable) and four parts of hydrochloric acid are gently warmed in a large flask (care being taken to avoid loss of liquid), until action ceases. While the solution is still warm, filter it through a weighed filter. Wash the residue with a little water, then dry and weigh it. This weight, corrected for the weight of the filter-paper and subtracted from the weight of original iron employed, will give the amount of iron dissolved.

For every 100 parts of iron thus dissolved add to the solution 260 parts of hydrochloric acid and 135 parts of nitric acid. Then warm the mixture in a glass flask on a water-bath until it has taken a reddish-brown color, and one drop, diluted with water, no longer gives a blue color with potassium-ferricyanide solution. The fluid is now evaporated in a weighed porcelain evaporating dish on a water-bath until 483 parts of the residual liquid contain 100 parts of iron by weight. This residual liquid is to be diluted and again evaporated to 483 parts until all nitric acid is removed. (The U. S. P. test for this is as follows: When a clear crystal of ferrous sulphate is added to a cooled mixture of equal volumes of an aqueous 10 per cent dilution of the solution and sulphuric acid, no brownish-black color should develop around the crystal (absence of nitric acid).) When the nitric acid-free condition is attained, dilute (before the solution has cooled) with distilled water so that it will weigh ten times as much as the iron dissolved in it. The fluid should be a clear, deep yellow-brown, and after dilution with water should give a white precipitate with silver nitrate and a deep blue with potassium ferrocyanide. The Ph. G. gives many tests for purity which are not necessary if C.P. materials are employed throughout the preparation. Preserve this fluid in tightly stoppered bottles, *protected from light*. This fluid can also be obtained when desired from any drug store. In America it will, of course, be made according to the U. S. P. formula, but I see no reason why this should make any essential difference in the result.

2. Raise to the boiling (certainly) point in an evaporating dish and add 25 cc. of the iron-chloride solution. The mixture is then boiled with stirring for two to five minutes more.

3. Cool (need not be completely cold) and filter. The precipitate thus obtained is allowed to dry somewhat and is then returned (together with the filter-paper) to the original evaporating dish (which should not be cleaned out and has meanwhile dried) and boiled in 200 cc. of 94 per cent alcohol. While the liquid is being stirred, fish out the filter-paper as it becomes freed of the precipitate.

4. After the solution has cooled, dilute 150 to 160 cc. with 96 per cent alcohol to make 200 to 210 cc. of the mixture.

5. Add 4 cc. of hydrochloric acid, and *use at once*. Use the stain only once.

Result. The result is a pale violet cell body, or, after the final alcohol treatment, a pale gray, while the trophosphonium stands out as a black-, or at least dark-violet.

On account of the variations in the fuchsin dye, it is necessary to arrive at the proper dilution of the mixture (step 4 above) by trial. If the stain is too strong, it may be advantageous to counterstain with alcoholic borax carmine.

The trichlorolactic method (Holmgren, '02 c and f: see also '02 b, d, and e)

This method is an improvement upon the preceding one, trichloroacetic acid being replaced as a fixative by trichlorolactic acid, which is said to be much superior.

1. Fix for twenty-four hours in 2.5 to 5 per cent trichlorolactic acid. In smaller animals a 2.5 per cent solution is best.

2. Run up and embed exactly as in step 2 of the trichloroacetic method, beginning dehydration in 50 per cent alcohol.

3. Sections 3μ to 5μ thick are stained for twenty-four to forty-eight hours in Weigert's resorcin-fuchsin solution. The proper dilution (see preceding notes) must be found by trial, on account of the variability of the dyes.

4. After twenty-four hours a preparation is removed from the stain, washed in 96 per cent alcohol, and examined with low power. If it is still pale, return it to the stain for another twenty-four hours. Then, as a rule, they are well stained.

5. After washing in alcohol, pass through xylol to balsam. The stain is said to be very permanent and easy to obtain.

After washing in 96 per cent alcohol (step 4) Holmgren ('02 f) sometimes finds it an advantage to place the slide in $\frac{1}{2}$ per cent chromic acid for an hour or more. The sections may thereby become clearer and more brilliant.

Supplementary notes

Holmgren ('02 a) suggests that trichlorpropionic acid might be still better than trichloracetic, but the matter is not again touched on. In the same place he states that a saturated solution of salicylic acid in 'Drittelalkohol' also yields satisfactory results. Later ('04), he also suggests 5 per cent trichlorlactic plus 5 per cent hydrochloric acid as a fixing agent. At the same time he suggests that the bad effects of the swelling of collagenous tissue may be in some cases offset by adding to the trichlorlactic-acid fixative, 1 per cent osmic acid in the proportion of 5 parts osmic to 100 of the fixing solution. Holmgren ('02 a) suggests that the best objects for trials are the spinal ganglia of kittens and young rabbits.

Many authors have tried Holmgren's methods, some arriving at a conclusion in essential harmony with his interpretations, others deciding that the trophosphonium is the Golgi apparatus pure and simple. Thus, Weigl ('10 a) and Cowdry have made osmic preparations to show the position of the Golgi material and then, by bleaching and staining with Fe-hematoxylin, have brought out the same structures as clear canals on a darkened cytoplasm (negative image), while Penfield ('21), doing the same thing with Cajal preparations, finds that the canals thus revealed do not correspond with the Golgi apparatus as blackened by silver nitrate. Cowdry ('21) also finds that in leucocytes preserved in formalin fixatives canaliculi may be visible which are without relation to the Golgi material. A few instances of the use by other workers of the more common fixatives and stains and then of Holmgren's special methods will be cited.

General methods of fixation and staining. These methods in the great majority of cases yield clear canals which are

rendered visible by the general staining of the cytoplasmic background—so-called negative images—as already noted.

Studnička ('99), nerve cells of lower vertebrates fixed in sublimate-acetic, vom Rath's picro-osmic, and Perenyi, stained with hematoxylin-eosin, methylene blue-eosin, or Fe-hematoxylin.

Bochenek ('01 a and b) uses Apáthy's method of Nachvergolding to stain neuroglial cells which send processes into actual nerve cells (*Helix*). The complete technic is given in his paper of '01 a. This result is clearly of no interest in connection with the Golgi apparatus, but in a comprehensive study of the trophospongial problem might be very usefully developed.

v. Smirnow ('01), nerve cells fixed in 1 per cent osmic acid, strong Flemming, Rabl, 3 per cent formalin, or 70 per cent alcohol, followed by Fe-hematoxylin or anilin dyes, such as safranin, Blue CLB, fuchsin D IV, and Neuvictoria. He also fixed in a mixture of 1 per cent osmic acid and 5 per cent potassium bichromate and proceeded as for Golgi's chrome-silver method.

Sjövall ('01), nerve cells fixed in formalin, stained with Fe-hematoxylin-erythrosin.

Retzius ('01 and '02), bone marrow of cats and rabbits fixed in Rabl, Carnoy, sublimate-acetic or sublimate-acetic-picric, stained with Fe-hematoxylin and erythrosin, acid fuchsin or toluidin.

Pewsner-Neufeld ('03), cyprinid taste organs by the method of Bethe (part IV), and also fixation in 10 per cent nitric acid according to a special method devised by Benda (part IV, p. 375).

Passek ('04), nerve cells, with a special method for Holmgren's canals:

1. Fix for five to seven hours in:

1 per cent Osmic acid in a saturated	
mercuric-chloride solution,	5 cc.
5.5 per cent Acetic acid,	10 cc.

2. Place twenty minutes in $\frac{3}{4}$ per cent solution of formalin.
3. Cut frozen sections and place for several minutes in 30 per cent alcohol iodized to remove the sublimate.
4. Then in pure 30 per cent alcohol to remove the iodine.
5. Wash in water and stain with Grenacher's hematoxylin. Peripheral sections should be treated with tannin, the central ones need not be.

Henschen ('04), nerve cells, fixed in sublimate-pieric or Carnoy, stained with Fe-hematoxylin-fuchsin-orange, toluidin-erythrosin, thiazinrot-toluidin, or fuchselin.

v. Bergen ('04), vertebrate tissues fixed in Kopsch's bichromate-formalin, stained with Benda's crystal violet.

Legendre ('05), nerve cells of *Helix* fixed in Lindsay Johnson or liquid D of Laguesse, stained with safranin-light green.

Reis and Nussbaum ('05), gas glands of fishes fixed in sublimate or sublimate with acetic or nitric acid, stained with Fe-hematoxylin and several counterstains, or erythrosin-toluidin blue, Ehrlich's triacid stain, etc.

Bouin and Ancel ('05), interstitial cells of the horse by the 'usual methods.' Said by the authors to be a fine object for the observation of the trophospongium.

Verson ('06), megakaryocytes fixed in Flemming, stained with Fe-hematoxylin.

Nemiloff ('08), nerve cells of fishes, fixed in Carnoy, alcohol-chloroform-acetic, pieric-sublimate, picro-nitric, or Holmgren's trichloroacetic acid with staining in fuchselin; but the best results with Lenhossék (not the pieric-acid mixture) and staining with Fe-hematoxylin.

Erhard ('10), liver duct of *Helix* and epididymis of the mouse fixed in half-concentrated sublimate with 2 per cent acetic acid or Zenker, stained with Weigert, Fe-hematoxylin, and many other common stains.

Weigl ('10 a), vertebrate nerve cells, fixed in formalin, Müller-formalin, Carnoy, sublimate, Flemming, or acetone, stained with many dyes, especially Fe-hematoxylin and Benda.

Bensley ('11), pancreas fixed in formalin-Zenker, stained with some basic dye. (See also Bensley, '10, and on plant material, part VI.)

Cowdry ('12), nerve cells of the pigeon with many fixatives—negative images.

Ross ('15), nerve cells of the crayfish fixed in Bensley's acetic-osmic-bichromate with acid fuchsin, or Zenker with Mallory's connective-tissue stain.

Trichloracetic or trichlorlactic acid methods. In general, these methods yield positively stained pictures of the trophospongium, and not negative images as in the case of the preceding group of fixatives.

v. Bergen, vertebrate tissues with 3 per cent trichloracetic acid and fuchselin, or with the same fixation and staining in Fe-hematoxylin or Benda's stain for central bodies.

Passek ('04), nerve cells, fixed in a modified fluid which he finds to eliminate the diluting qualities of trichlorlactic acid alone:

1. Fix for twenty hours in:

Trichlorlactic acid,	10 parts
Formalin,	10 parts
Acetone, C.P.,	8 parts

2. Place in pure acetone for two to three hours and embed in paraffin.

3. Stain in:

Thionin,	1 cc.
Phenol,	0.5 cc.
Alcohol,	10 cc.
Distilled water,	89.5 cc.

4. Differentiate in 1 per cent aqueous solution of eosin. Then it is well to stain in a very strong aqueous solution of safranin, toluidin blue, or Bismarck brown.

5. Then wash in water, alcohol, oil of cajeput, and balsam.

A second variation is also given by Passek ('04) as follows:

1. Fix for one and one-half to two and one-half hours in:

Trichlorlactic acid,	1 cc.
Acetone,	20 cc.
Ether,	10 cc.
Formalin,	2 cc.

2. Place in chloroform for one and one-quarter hours and thence into paraffin at 52°.

3. Stain as before.

Erhard ('10), liver duct of *Helix* and epididymis of the mouse, fixed in trichlorlactic acid of 3½, 6, and 8 per cent, stained with Fe-hematoxylin, Delafield, Weigert, safranin, and many other stains.

Weigl ('10 a) prepared vertebrate nerve cells by the method of Kopsch (part III) and stained with fuchselin (see section in this paper on the idiosomic component, p. 107, and also Weigl, '10 b). Weigl ('10 a) also fixed in osmic acid or osmic-containing mixtures, for example, sublimate-osmic, trichlorlactic acid-osmic, Flemming, and also in Rath and Golgi, followed by staining in fuchselin according to Holmgren's procedure. He states that positive pictures of the Golgi network (trophospongium) thus result, which are secured not only more easily and constantly, but are also much more beautiful and intense than the stain which follows the simple trichlorlactic-acid fixation as recommended by Holmgren.

Poluszyński ('11), crustacean nerve cells fixed in trichlorlactic acid with acetic or osmic acid, stained with fuchselin.

Cowdry ('12), nerve cells of the pigeon by Holmgren's special methods, which, he states, stain the Golgi apparatus rather irregularly even in a single cell.

In concluding this treatment of the technic for demonstrating the trophospongium and canals of Holmgren, it should again be emphasized that while the special methods with fuchselin staining yield a *positive* stain of the trophospongium, the more general methods usually yield only *negative* images of canal-like structures (p. 116).

THE LACUNOME

In a series of recent papers Corti has developed the thesis that the Golgi apparatus is essentially a collection of lacunae (or vacuoles) constituting what he terms the 'lacunoma' (lacunome). This interpretation joins, on the one hand, with Holmgren's theory of the trophosphongial canals and, on the other, with the vacuome theory as developed in somewhat different directions by Parat and Guilliermond (see section on the vacuome, part VI). The methods used by Corti are like those used by other workers, only in their interpretation is there a difference.

Corti ('20 a) uses the arsenious-acid method of Golgi followed by Veratti's treatment and staining with carmalum. He mounts directly after the final washing in water, in Apáthy's gum-and-syrup medium. In a later paper ('20 b), fixation in Tellyesniczky and Maximow is recommended. In 1924, Corti suggests fixation in Meves or F. W. A. for several days and then postosmication for ten days in osmic acid (0.5 to 1 per cent); also Golgi's arsenious-acid mixture (fixation for one to two to four hours with intestine of guinea-pig) and Cajal's formol-uranium nitrate method (fixation for two up to seven to nine hours). Later still ('25) he uses fixation in sublimate, Dominici, Maximow-Levi, Flemming, Altmann, Regaud (with postchroming), and staining with Fe-hematoxylin (also with prolonged mordanting with heat).

It will be observed that with most of these methods (except, of course, the silver ones) negative images are to be expected, at least in somatic tissues of vertebrates.

CHROMIDIA AND THE GOLGI APPARATUS

At the time of the promulgation of the chromidial hypothesis it was generally held by its adherents that the so-called Golgi structures were really only a part of the all-inclusive chromidia. As such, the Golgi apparatus was studied by several workers on chromidia, the methods used being for the most part those already described in these papers as coming under the head of general fixation and staining methods (see especially part IV and under 'Trophosphonium' of this paper). In most cases, however, the fixatives

were hardly adapted for the proper preservation of Golgi material, and the results are largely fragmentary and problematical.

The most clear-cut case is that of the liver duct in *Helix* and the epididymis of the mouse studied by Erhard ('10). For his methods see under the 'Trophospongium' (pp. 123 and 125). Goldschmidt ('04) used sublimate mixtures particularly, with various stains. See also Popoff ('06 and '07), and various papers of Jörgenssen (e.g., '10), etc.

CRITIQUE AND SUGGESTIONS FOR PRACTICE

The methods considered in this paper are, in general, much inferior to the more specialized methods for demonstrating the Golgi apparatus. So much so, indeed, that one would scarcely have recourse to them except for studies of a very special nature. For routine work and for the general student, therefore, these methods are of little interest, but they nevertheless contain the solution to the old dispute as to the nature and homologies of Holmgren's trophospongium and the details of Golgi-apparatus structure. Sooner or later, these problems must be made special objects of a more sustained attack than has hitherto been attempted. The nature of the idiosomic substance, the chemistry of the Golgi material, the occurrence of intracellular canals and vacuoles, the penetration of one cell by protoplasmic branches from another cell—all these questions have become inextricably entangled with the problem of the Golgi apparatus proper. To the methods summarized in this paper we must look for at least those preliminary steps which will be necessary by way of orientation with the problems as they have been shaped by previous study.

As to methods for practice, it seems of doubtful value to try any of those here described except as one wishes to use them for serious investigation. Of them all the most promising would seem to be the special methods devised by Holmgren (pp. 118 and 120).⁹ Their further application to the

⁹ See page 121 for Holmgren's suggestions as to material which will give the best results.

Golgi problem along the lines already tried by Weigl ('10 a) and Kopsch ('26) would seem to promise much in the better crystallization of opinion concerning the structure of the Golgi apparatus—a problem just now of much importance.

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THE ORIGIN AND FATE OF POLYNUCLEATE CELLS

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FOUR FIGURES

INTRODUCTION

Polynucleate cells are so widely distributed under normal and pathological conditions and so much fundamental significance is commonly assigned to them, that we need to know fully their method of origin and ultimate fate.

Following the observation of Robin(1) that polynucleate cells clearly differed from the large mononucleate forms, various theories were presented to account for the occurrence of the former. Some of these were highly speculative(2); others were based on sound observation(3). Although Arey (4) lists seven theories, and while Liénaux and Hamoir(5) have added another, the body of evidence permits of a three-fold classification of earlier views: that the polynucleate cells are produced by a fusion of simpler units(6); by a nuclear division without an accompanying division of cytoplasm(7); or, sometimes by fusion and at other times by nuclear division(8).

The variety of opinions as to the origin of polynucleate cells is inherent in the method hitherto used in studying the problem. While the fixing and staining of cells has been of the utmost importance in elucidating their structure, function, and probable relationship, other methods are needed to determine their genesis. The truth as to the origin of polynucleate cells can be obtained only by a careful pedigree study of these cells in pure culture. Such an ideal is difficult of attainment(9) but the recent work(10) with tissue cultures augurs well in that direction.

EXPERIMENTAL PRODUCTION OF POLYNUCLEATE CELLS

In a previous communication(11) methods were detailed for a genetic study of the protozoan, *Amoeba proteus*. Occasionally, large forms with two, three, and four nuclei appeared in isolated and mass cultures of pure lines of the simple mononucleate amoebae. Subsequent study failed to reveal any signs of either spontaneous or experimental fusion of mononucleate cells. Nor could evidence be obtained for the view that the additional nuclei were due to the persistence of nuclei of ingested cells.

While studying the division process in amoeba, one is impressed by the interval of fifteen to thirty minutes that elapses before the two daughter cells are produced. During

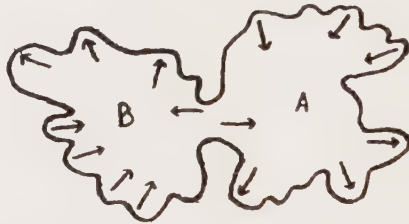


Fig. 1 Camera-lucida outline of a dividing mononucleate amoeba. The arrows point in the direction of the protoplasmic flow.

this period there is a constant and rhythmic reversibility in the streaming of the protoplasm between the dividing parts (fig. 1). The granules can be seen moving first into one part, *A*, then into the other, *B*, and vice versa. In the meantime the two amoebae slowly recede from one another, narrowing the connecting strand between them, until it snaps. But if before this occurs, a simple experiment is performed, the cytoplasmic division is profoundly altered. A dividing amoeba (fig. 1) is transferred by means of a fine capillary pipette to the periphery of a drop of culture fluid (0.5 mm. in diameter) which has been placed previously on a glass slide. The organism is so oriented (fig. 2) that one of the dividing cells, *B*, fits snugly into the drop formed by the minute quantity of fluid that accompanied the transfer. For

a moment or two, the usual alternating rhythm of granular movement is observed. But when a pseudopod of the portion *B* reaches the edge of the drop, it is quickly withdrawn and a greater flow of granules proceeds into *A*. As the smaller drop evaporates, its edge approaches *B* and prevents it from projecting any more pseudopods. The only part of *B* that is not affected by the evaporating drop is the portion attached to the connecting strand. The alternating movement of the

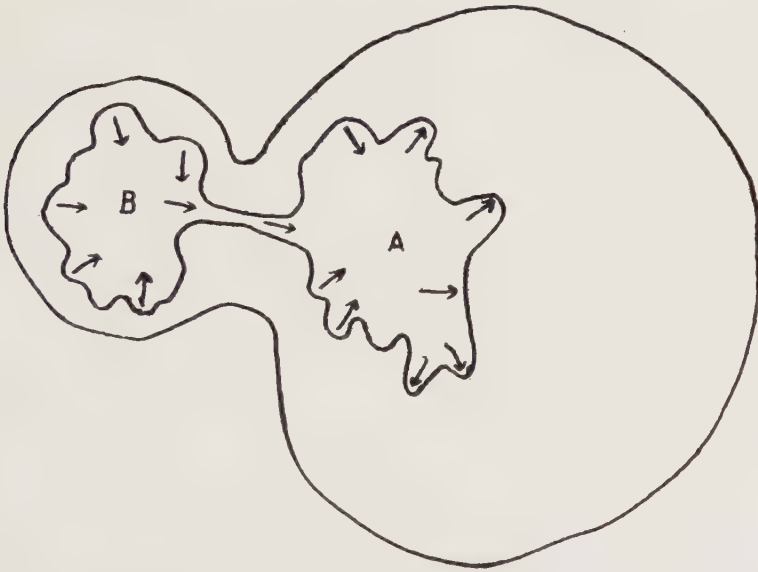


Fig. 2 Diagram of a dividing amoeba oriented in two unequal drops of fluid. The arrows indicate the direction of the protoplasmic flow as the fluid evaporates.

cytoplasm becomes unequalized, *A* receiving a more forceful and longer flow. Further evaporation of the fluid surrounding *B* virtually forces its protoplasm to proceed with increased rapidity into *A*. This streaming of one part into the other continues as long as the movement of *B* in any other direction is prevented. Eventually, all of *B* flows into *A* and the resultant amoeba does not attempt to divide again. Subsequent examination reveals that an organism is produced which is similar to the binucleate cells found in the pure-line cultures.

Following an average interval of seven days, these binucleate forms divide into two or more parts. When the above experiment is performed on a binucleate amoeba dividing into two parts, a tetranucleate organism may be obtained. But the most common method of reproduction is an unequal division of the cytoplasm into three parts. By the use of the evaporating-drop method, while the triple cytoplasmic division is in progress, one may obtain binucleate, trinucleate, or tetranucleate cells(11). The greatest difficulty is experienced in attempts to modify the binucleate amoeba that is dividing into four mononucleate portions. The slightest manipulation causes the separation of the parts, and every experiment performed to control the division proved unsuccessful. Similar experiments with dividing trinucleate amoebae yield organisms with four and six nuclei. In an attempt to modify the division of a tetranucleate form that was dividing into two unequal parts, a hexanucleate amoeba was obtained. The high mortality among the tetranucleate and hexanucleate forms and the relative rarity of observations on division among the survivals render further study of the process of increasing the number of nuclei in cells extremely difficult.

It is interesting to note that Weigert(12) suggested that the genesis of polynucleate cells in tuberculosis, syphilis, and actinomycosis was due to pressure phenomena, either of a chemical, mechanical, or biological nature. Chabry(13) was able to produce a binucleate cell by pressing with a sharp needle on a dividing ovum of *Ascidia asperia*. Buxton(14) claimed that imperfect cell division could result from the fact that tissue cells are not bathed in lymph from all sides. The introduction of foreign bodies into tissues could also act as one of the factors in making for imperfect cell division. But the researches of Forbes(15) and Lambert(16) indicate that foreign-body giant cells are formed by the fusion of mononuclear elements.

The evidence shows that polynucleate forms of *Amoeba proteus* are formed only by the failure of the cytoplasm to separate after the nucleus has divided. It is suggested that

polynucleate cells in normal and pathological tissues arise by a similar mechanism.

THE FATE OF POLYNUCLEATE CELLS

While the many possible sources of origin of polynucleate cells have been carefully and repeatedly studied, the ultimate fate of these cells has received relatively scant attention. Yet enough work has been done to permit Arey(4) to list six theories, showing the great divergence of views. Again, as in the study of the genesis of these cells, absolute certainty as to what becomes of them can be obtained only through a long-continued study on the living cell.

TABLE 1

Showing the difference in viability between the mononucleate and polynucleate amoebae

CHARACTER OF CELL	NUMBER OF AMOEBAE STUDIED IN PURE- LINE CULTURE	NUMBER OF AMOEBAE THAT DISINTEGRATED BEFORE DIVIDING	MORTALITY RATE
			<i>Per cent</i>
Mononucleate	1472	89	6.2
Binucleate	310	34	10.9
Trinucleate	49	12	24.3
Tetranucleate	16	7	43.7
Hexanucleate	5	4	80.

In a consideration of the fate of the polynucleate amoebae found in the pedigree cultures and produced experimentally, one is struck by the decreased viability of these cells. The organisms, in addition to containing a greater concentration of cytoplasmic material, react differently to the culture that was used for the mononucleate cells. In a constant culture medium and in the presence of an overabundance of food, many of the more complex forms lead a sluggish existence for days (in one case, forty days), seldom feed, and finally disintegrate. A study of the figures in table 1 shows that as the number of nuclei in the cell increases, the organism has less chance of dividing. The small number of amoebae with more than two nuclei that were available for study make a definite comparison of percentages impossible. But even from the figures as given, together with observations

on the change in behavior of the organisms themselves, one cannot escape the conclusion, that an increase in nuclear content lowers the vitality of amoebae.

The remainder of the polynucleate forms divide in a variety of ways(11, 17). These seems to be no limit to the methods of division of these cells. With an increase in the number of nuclei in the mother form, the nuclear character of the progeny becomes more diverse. But the amoebae remain in the polynucleate state only temporarily. As a rule, all the progeny of a given line of polynucleate forms become mononucleate again in three generations. Yet, occasionally, one meets with a line which has polynucleate progeny for many more generations. The ancestors of such a line (fig. 3) had been simple mononucleate amoebae in pure-line culture for more than a year. Following the appearance of the original

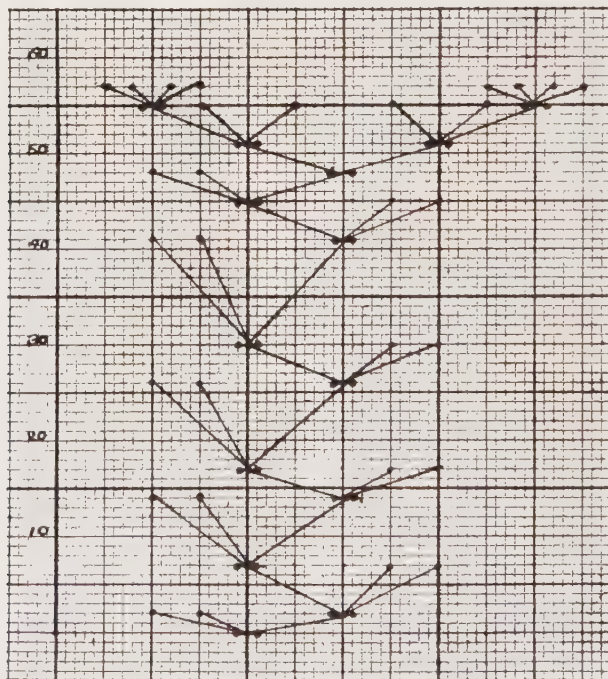


Fig. 3 Genealogy of a binucleate amoeba. Each line represents one of the progeny. The dots at the end of each line represent the number of nuclei in that organism. The ordinates represent days between generations.

binucleate form, eleven successive generations of binucleate cells were produced. It is remarkable that the first nine divisions were alike, namely, in each generation there was a triploid division of the cell into one binucleate and two mononucleate parts. The tenth division resulted in two binucleate amoebae. Each of these in the next generation again gave a threefold division. The line became entirely mononucleate in the twelfth generation, when each of the binucleate cells divided into four mononucleate parts.

Study of the genealogy of a trinucleate amoeba (fig. 4) shows that it continued as a trinucleate cell for only two

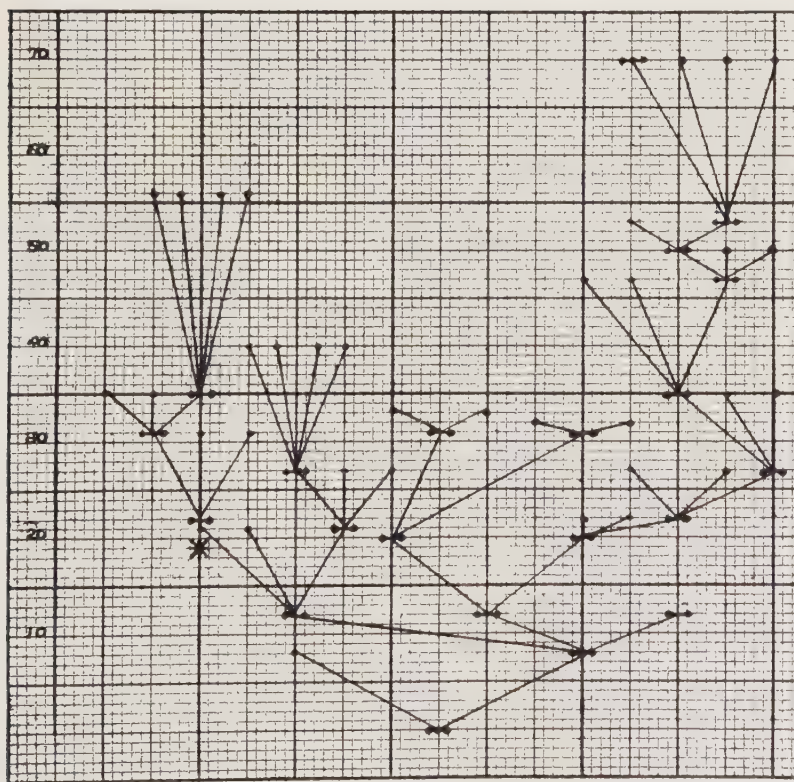


Fig. 4 Genealogy of a trinucleate amoeba. Each line represents one of the progeny. The dots at the end of each line represent the number of nuclei in that organism. The ordinates represent days between generations. * This mononucleate cell was prevented from dividing, resulting in a binucleate organism.

generations. In the next three generations several collateral lines of binucleate progeny existed. An additional binucleate line was started when one of the mononucleate cells of the fourth generation was prevented from dividing by the evaporating-drop method. This line persisted as a binucleate cell through two generations, each time giving a triploid division into a binucleate and two mononucleate amoebae. But the polynucleate career of this line ended in the next generation, when the binucleate amoeba divided into four mononucleate cells. The figure also shows that one of the binucleate collaterals divided into three parts for six generations, each division having as one of its products a binucleate form. In the tenth generation a trinucleate amoeba was produced. Again, as at the beginning of the line, there were only two generations of the trinucleate cell. With the disintegration of this form twenty-six days later, the line ended, having produced four trinucleate, seventeen binucleate, and thirty-nine mononucleate cells.

The figures, representing only two of the many different types of family trees that were obtained, give an adequate idea of the fate of polynucleate amoebae. They show that the polynucleate forms tend to reproduce in such a manner that the great majority of the progeny revert to the simple mononucleate units; a few remain like the parent form; while occasionally a more complex form is produced. It is suggested that the polynucleate cells occurring in tissues have a similar fate, namely, a very large percentage disintegrate (18), the remainder showing varied methods of division, into more complex and simpler units(19), until the original mononuclear structure is reached.

CONCLUSIONS

1. Experimental evidence is presented which shows that polynucleate cells arise by nuclear division without an accompanying division of the cytoplasm.

2. While many of these cells finally disintegrate, some retain their polynucleate nature through several generations, ultimately reverting to the mononucleate form.

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ON THE LYMPHATIC VESSELS OF SCYLLIUM CANICULA

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The lymphatic system of teleost and ganoid fishes is well known through the works of such authors as Jourdaine, Trois, Hopkins, Allen, McClure, Hoyer and Michalski. But statements regarding the position of the lymphatic vessels in elasmobranchs, made by Monro, Fohman, Robin, and Sappey, are so contradictory that Paul Mayer was led to ask, in 1917, whether the elasmobranchs have any lymphatic vessels at all.

These contradictions of opinion have arisen from the fact that some authors have identified certain vessels (or tracts of vessels) as lymphatics, while others have considered them blood vessels.

The cause of these disagreements has been two-fold: 1) the method of research, namely, injections with quicksilver, which by its weight rends asunder the delicate vessels and passes from one system of vessels to another; and, 2) the assumption that results obtained with teleosts could also be ascribed to the elasmobranchs on the ground that the lymphatic system of both classes is identical. But, not later than 1887, T. J. Parker showed that the vessel which runs laterally in the elasmobranchs is a vein (as did also Hochstetter in 1888), while other authors have asserted that the vessel which runs quite similarly in teleosts and ganoids is a lymphatic vessel. These contradictions have made it necessary to investigate these vessels in elasmobranchs anew.

I began the first inquiry before the war, but the embryonic materials were both hastily gathered and insufficient. But

now in these last years, the researches carried out in the zoological stations in Villefranche and Roscoff on embryonic material as well as on adult specimens of *Scyllium canicula*, have enabled me to reach the following conclusions, which are presented below in the form of a preliminary statement.

The method which I used was that of injection with Prussian blue. Either the lymphatic vessels alone were injected or these were supplemented by injections of the veins or the arteries. In order to distinguish the lymphatics from other vessels, the veins were injected with India ink. The specimens were examined both after sectioning and in toto.

The principal collecting vessels in elasmobranchs run on either side of the aorta from the end of the tail to the head. Segments of these vessels have already been described several times, but not as lymphatic vessels. Thus P. Mayer described them as 'Vasa vasorum'; Grünfeldt, as 'Veins juxta-aortiques'; Vialleton, as veins of a lymphatic character, and Favaro, as 'Vasa intermedia.' Only Diamare affirms that they are lymphatic vessels, united directly with the lymphatic vessels of the intestines; but he, too, was unable to locate their entrance into the veins. However, as my researches have shown, these are really lymphatic vessels which collect the lymph from the intestines and muscles and run to the veins, as will be described below.

Although both Vialleton and Diamare showed the existence of lymphatic vessels in the intestines, they disagreed as to the terminations of the vessels. According to Vialleton, these intestinal rami unite to form the lymphatic plexus at the root of the mesentery; according to Diamare, they empty into the trunks which accompany the aorta. Yet both Vialleton and Diamare are right in their assertions, since the intestinal lymphatics do drain into the plexuses at the root of the mesentery and thence to the trunks near the aorta.

These principal longitudinal trunks also collect the lymph from the muscles of the body and tail, through little branches which accompany the small intersegmental arteries. They run with the little arteries and veins of the myosepta which

divide the muscle segments, and in the septa which separate the dorsal muscles from the ventral. Also I have been able to show a very thick network of lymphatics under the peritoneum and a still thicker one over the kidneys. All these vessels and networks pass on to the principal lymphatic trunks.

Since these two para-aortic trunks have the same course, termination and function as the thoracic ducts of other vertebrates, I have therefore defined them in elasmobranchs as thoracic ducts. Correspondingly, there are also jugular trunks, which run along the jugular vein, between this vein and the muscles and pass to the cardinal sinuses. Furthermore, at the place where the subclavian arteries branch from the aorta, there are two corresponding branches of the thoracic ducts which accompany these arteries and pass to the cardinal sinuses.

As regards the distribution of their branches, the thoracic ducts of elasmobranchs are much the same as in other fishes and in urodeles, except that in skates the lateral lymphatic trunks are replaced by lateral veins—a circumstance which has mystified a large number of investigators.

Finally, a study has been made of the development of the lymphatic system, in elasmobranchs, in the course of which it has been ascertained that the thoracic ducts first appear in 31-mm. embryos ('Q' stage of Balfour), at the level of the subclavian arteries, where they arise from the proliferating wall of the cardinal sinuses.

This work will appear 'in extenso' in the course of the year 1928 in the Bulletin of the Polish Academy of Science.

AN ANALYSIS OF THE REACTION OF THE HUMAN GALL BLADDER TO FOOD

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NINETEEN TEXT FIGURES AND ONE PLATE

Since the discovery that a meal of egg yolk and cream would completely empty the human gall bladder of its fluid contents (Boyden, '25) the writer has made a detailed study of the reaction of the gall bladder to a standardized meal in twenty-four normal individuals of the student class. In the first six cases, previously published ('26 b), x-rays were made at fifteen-minute intervals only, through fear that a greater total exposure to the Roentgen ray would produce an erythema. Since then it has been found possible to make a larger series of exposures at much shorter intervals after a test meal, thereby revealing some important features not previously observed. These, in turn, have led to experiments designed to test the nature of the factors which control the flow of bile from the biliary reservoir into the intestine, so that now it is possible to speak with some assurance regarding the normal response of the human gall bladder to the ingestion of food.

SEQUENCE OF EVENTS AFTER YOLK AND CREAM

Methods

In the last eighteen cases the following schedule was prescribed for each 'patient.' On the evening preceding the experiment (between 5 and 6 P.M.) a light meal was eaten, consisting of tea and toast (without butter, cream, or milk). One hour afterward, the patient began taking capsules of tetraiodophenolphthalein (Iodeikon) by mouth—four every half-

hour with water, until ten to sixteen were taken, depending on the patient's weight. No other food was eaten until after the first x-ray picture at 9 o'clock the next morning. A satisfactory gall-bladder shadow having been obtained, a standard meal of five egg yolks, well mixed with half a pint of cream, was prepared. This done, the fasting patient was again x-rayed, and immediately thereafter given the tumbler of yolk and cream to drink. The third picture was taken two minutes after the glass was raised to the lips (a minute and a half usually sufficing to swallow the whole mixture) following which, successive pictures were made at four, eight, twelve, sixteen, and twenty minutes postcibum and every five minutes thereafter up to at least an hour from the time of drinking the yolk and cream. In cases in which two or three hours were required to empty the gall bladder, pictures after the first hour were made at ten- or fifteen-minute intervals. The exposing and developing of the x-rays were done by the writer in order to secure uniformity of conditions. Especial care was taken in placing the patient in the same position each time, this being accomplished by centering the target over a pencil mark on the patient's back, each individual lying prone on the x-ray table throughout the duration of the experiment. Verification of the position was made possible by the fact that the x-ray table was equipped with an engraved cross which recorded the center of the target on each film. After the cholecystograms were traced, the changing volumes of the gall bladder were computed, according to the method described in the Appendix.

Condition of the gall bladder before the test meal

A description of the events following the ingestion of food necessarily begins with the period preceding a meal. At the end of the fifteen hours following oral administration of the dye, during which time the patient has refrained from eating, it is usually found that the gall bladder is not quiescent, but is either filling or contracting. In one patient x-rayed at twenty-minute intervals for four hours by the writer, one

such spontaneous contraction was recorded about the middle and another at the end of this period ('26 c); and frequently it has happened that when several exposures were made preceding a meal the volume of the gall bladder was found to be either increasing or diminishing. Some of these spontaneous contractions are very pronounced and result in a considerable discharge of bile (*series 3*, fig. 17). This probably accounts for the somewhat alarming statement that appears on the labels of a well-known and otherwise satisfactory commercial preparation of tetraiodophenolphthalein.

It is important that on the morning the pictures are to be taken the patient be shut off from appetizing odors of food, especially that of broiled or fried meats. The gall bladder responds markedly to psychic influences and is quickly emptied by the smell or thoughts of tempting foods. Many pictures have thus been spoiled, the gall bladder has emptied and a shadow is not obtained.

To see whether such a 'cephalic' phase of evacuation could really be demonstrated, the writer suddenly placed a dish of fried bacon before a patient with whom he had been working. During the ten minutes in which she continued to smell this savory comestible she succeeded in developing a ravenous appetite. But apparently the gall bladder was on the 'up phase,' for its volume increased during this interval and for twenty-three minutes thereafter (fig. 1). However, in less than two minutes after the same patient drank five egg yolks, the gall-bladder shadow abruptly diminished, reaching a volume as low as 1.6 cc. before the x-rays were discontinued. Subsequent attempts with other individuals (fig. 16) showed that in seven out of eleven cases there was a slight response to the smell of food, but that this method of stimulating the release of bladder bile is negligible (compare p. 173). Therefore, those spontaneous contractions of the gall bladder which occur during periods when there is no food in the stomach cannot be attributed to olfactory or psychic stimulation.

The initial response

Perhaps the most striking feature of the reaction of the gall bladder to food is the very short latent period of contraction, for in fourteen out of seventeen patients in which x-rays were made at two and four minutes postcibum the gall bladder showed a marked diminution of volume within the first two

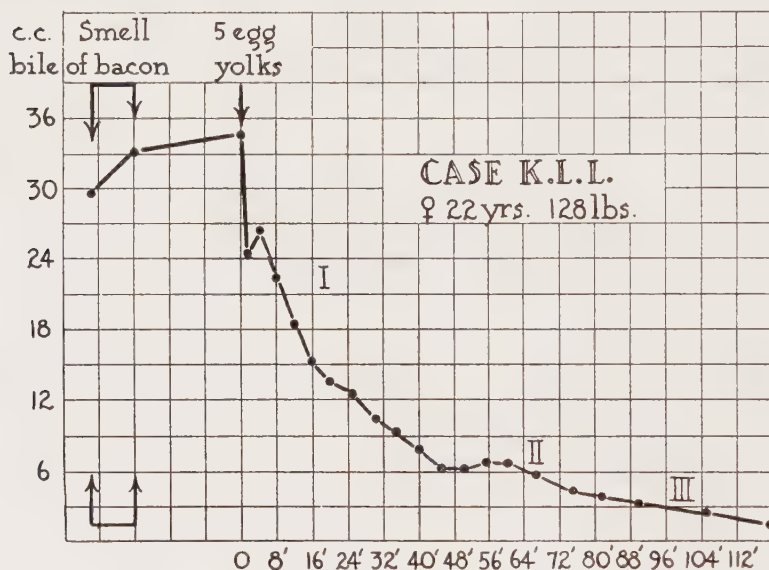


Fig. 1 Contraction curve, showing the reduction in volume of the human gall bladder after a meal of five egg yolks mixed with water. The bracketed arrows indicate period of ten minutes during which the patient continuously smelled a dish of hot fried bacon. 15°, fifteen hours after oral administration of tetraiodophenolphthalein; 8', 16', etc., minutes postcibum; I to III, phases of contraction (compare also with *K. L. L.*, table 1).

minutes after food entered the mouth (fig. 2). In the only case in which the first two x-rays were taken at one-minute intervals (*L. O. M.*, fig. 3), no diminution of volume occurred during the first minute. In another case in which the first picture was taken at one and one-half minutes postcibum (*L. R. F. (milk)*, fig. 3) the gall bladder had begun to contract before this time. Since fluoroscopic observation indicates that the 'head' of a meal of egg yolk passes the pylorus a few sec-

onds after deglutition, the latent period of the human gall bladder is therefore established as approximately one minute after egg yolk enters the duodenum.

This corresponds exactly to the latent period established by McMaster and Elman for the gall bladder of the dog, in

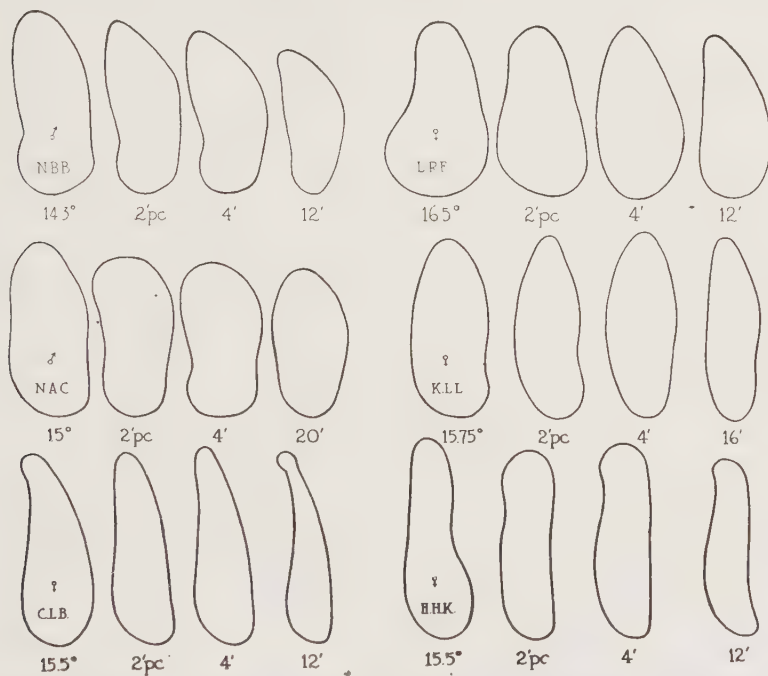


Fig. 2 Tracings of the first four cholecystograms from six x-ray series, illustrating the change in shape of the human gall bladder two minutes after the ingestion of five egg yolks and half a pint of cream (2' pc). In each series note narrowing of fundus. The contraction curve for each case may be found in figures 3 and 4, under the appropriate initials (compare also with data in tables 1 and 2).

which animal the initial response, as recorded by a manometer directly connected with the cystic duct, consisted of a rise in intravesicular pressure about one minute after eating (fig. 11)—an occurrence which has been correctly interpreted by these authors as due to the contraction of the gall bladder. That the initial diminution in volume of the human gall blad-

der is likewise due to contraction of the organ is evidenced by the change in shape which it undergoes in the first two minutes after a meal. Six typical cases are shown in figure 2. In each instance the fundus of the organ becomes narrower, and in several cases the relative proportions of the body and fundus are reversed—as if the bile were being compressed from below, causing the body and neck of the organ to bulge (e.g., 2' *pc*, cases *N. A. C.* and *H. H. K.*, fig. 2). But if the gall bladder were merely a passive organ, the first change in shape would be a shrinkage of its upper portion following the loss of bile from the region nearest the cystic duct.¹ Similar

¹ Since the significance of this change in shape was first noted ('26 a), much supplementary evidence has been advanced by the writer and others to establish the fact that bladder bile is expelled primarily by the force of the intrinsic musculature of the gall bladder—that musculature which has been described by Halpert and by Pfuhl as an hypertrophied *muscularis mucosae*. Credit for this demonstration belongs chiefly to three groups of American investigators working independently and publishing their results in the summer of 1926, namely, Higgins and Mann, of the Mayo Foundation; McMaster and Elman, of the Rockefeller Institute, and both Whitaker and the writer at the Harvard Medical School. (For résumé of this evidence, see Boyden, '28 a). If more evidence were needed, it has since been provided by the outstanding demonstration of Doctor Higgins at the April meetings of the American Association of Anatomists. There he presented moving pictures of vigorous peristalsis of the gall bladder induced by a meal of egg yolk in the teleost fish, *Ameiurus nebulosus*. This striking observation provides us with a new perspective of the evolution of the gall bladder in vertebrates. At one end of the series (fishes), the organ appears as a well-developed muscular reservoir which empties its contents after food by rhythmic movements as pronounced as those of intestinal peristalsis. At the other end of the series it presents a variety of conditions. In some twenty or more species of mammals and birds it has entirely disappeared; in others, as the pigeon, it is present in the embryo only. In a third group, as the two-toed sloth (Halpert, '27) the gall bladder persists in adult stages, but its musculature has almost disappeared. Physiologically, there is just as great a diversity. In birds, peristalsis of the duct system still persists (Claude Bernard), but in mammals peristalsis of the gall bladder has been replaced by a more or less concerted action of the whole *tunica muscularis*. But even here there is a difference in action, the mechanism of the expulsion of bladder bile being such that in the rabbit and dog, for instance, the gall bladder is only partially emptied after a meal; whereas in the cat, guinea-pig, and man it is so effective that the vesicle can be completely emptied of its fluid contents. This great diversity of action in mammals, therefore, makes it imperative that generalizations based on experiments with the rabbit and dog should not be extended, without qualification, to the human gall bladder.

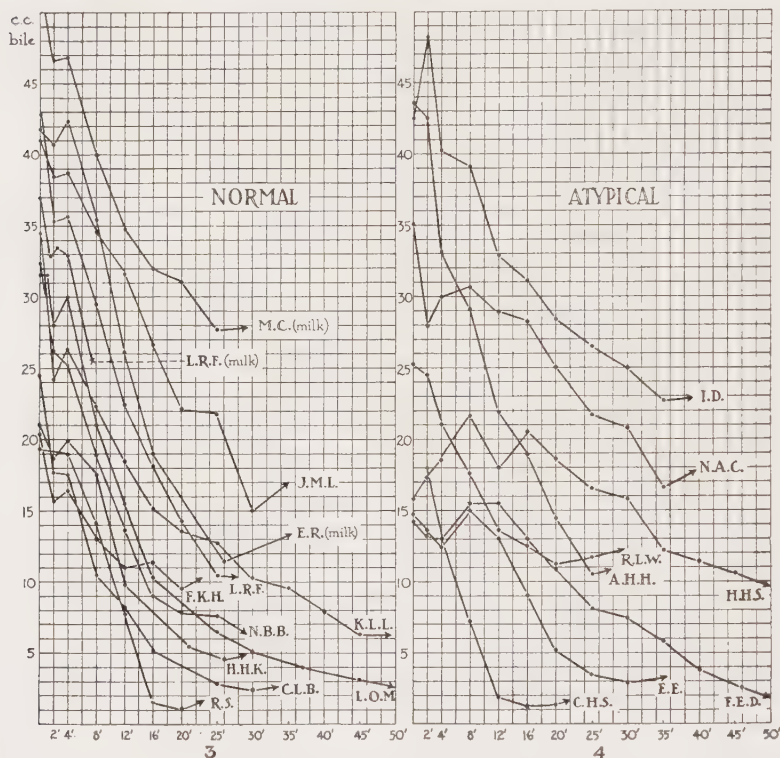
changes in shape of the human gall bladder have been noted by Schöndube ('27), in a paper recording three x-ray series, two of which showed the response to egg yolk. He notes that at first a contraction appeared in the transverse, later in the longitudinal direction, and that the erection and broadening of the neck could be demonstrated. Incidentally, we are indebted to Schöndube for first calling attention to the visualization of the common and hepatic bile ducts, for which purpose he used very thin subjects.

A second observation that can be made about this first period of contraction, as recorded by the curves in figure 3, is that the diminution of volume in the first two minutes is usually greater than that of any subsequent two minutes—an average of 5.1 cc. as compared with 3.1 cc. in the ten cases shown in figure 3 (omitting the three 'milk' series and *R. S.*). An interpretation of this will be found on page 169.

The two-minute pause

At the end of the first two minutes, the descent of the curve is abruptly checked and is not resumed again until at least two minutes later (fig. 3). This has occurred exactly at this point in so many cases (in twelve out of the seventeen patients who were x-rayed at two and four minutes after egg yolk) that it cannot be considered an artifact, but a very characteristic and significant feature in the emptying of the human gall bladder. Nine of the twelve cases (fig. 3) are quite similar and probably represent the normal situation. The remaining three exhibit some aberrance in time relations, and for that reason have been placed with the atypical forms shown in figure 4. Thus in two of these (*F. E. D.* and *E. E.*) the 'first response' has a duration of four minutes instead of two. Also in these two cases and in *N. A. C.*, the 'two-minute pause' is lengthened to four, six, or eight minutes before the principal period of contraction gets under way. To these twelve cases should also be added three more (viz., *A. H. H.*, *R. L. W.*, and probably *C. H. S.*, fig. 4), which merely differ from the others in the apparent absence of a 'two-minute pause.' But since

we have seen that the latter interval can be lengthened to four or even eight minutes, it is equally possible that it could be reduced to the point where it would not be recorded in



Figs. 3 and 4 Contraction curves of the human gall bladder, illustrating the first phase of contraction after ingestion of egg yolk and cream in seventeen students. For additional data see appropriate initials in tables 1 and 2. Cases *M. C. (milk)*, *E. R. (milk)*, and *L. R. F. (milk)* are added for purposes of comparison. Case *L. R. F. (milk)* shows the beginning of the curve only. Ordinates, cubic centimeters of bile in body and fundus of the gall bladder. Abscissas, minutes after ingestion of yolk and cream. All curves in figure 3 show the characteristic 'two-minute pause' (see text).

x-rays taken at merely two-minute intervals. There remains the further possibility that, since it depends on the interaction of gall bladder and sphincter (as will be pointed out later), its absence may be due to occasional lack of coincidence of action of these two organs.

In contrast to the fifteen cases just described, which may all be grouped together as lying within narrow limits of variation, are two cases which are decidedly atypical (*I. D.* and *H. H. S.*, fig. 4). In these two individuals, the emptying of the gall bladder is preceded by a period of filling. Since these cases require interpretation, discussion of them will be postponed to a later section of the paper (p. 181).

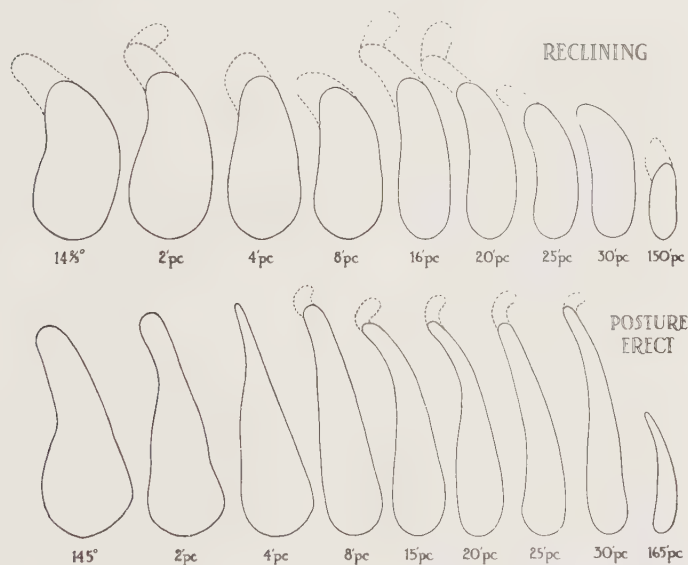


Fig. 5 Selected cholecystograms, showing the negligible effect of gravity on the emptying of the gall bladder of a young woman (fig. 6).

The principal period of discharge

By this phrase is meant that portion of the first phase of contraction which results in the greatest discharge of bladder bile. It follows immediately after the two preliminary features already noted—the ‘initial response’ and the ‘two-minute pause’—and ends anywhere from sixteen minutes to sixty minutes postcibum. In the last seventeen cases after egg yolk (figs. 3 and 4) in which the first phase was clearly marked off from the succeeding phases, its average duration was thirty-two minutes, during which time the contents of

the gall bladder (excluding the neck) was reduced approximately three-fourths of its volume (averaging 66 per cent in the six men and 80 per cent in the eleven women, the extremes ranging from 55 per cent to 93 per cent in men, and from 60 to 94 per cent in women). From these data it must be apparent that the first phase is the most significant portion of the cycle of emptying and filling, for by means of this a large

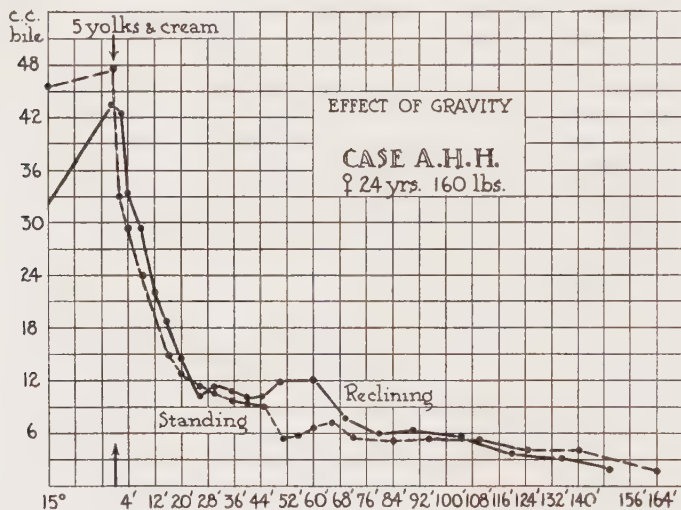


Fig. 6 Contraction curves of the two cases shown in figure 5. For additional data see *A. H. H.* (table 1 and fig. 3). Two similar series, made upon a young man (fig. 3, Boyden, '28 a), would seem to establish, 1) the constancy of the response of the human gall bladder to the same stimulus when repeatedly applied to the same individual; 2) the negligible effect of gravity on the emptying of the gall bladder.

amount of concentrated bile is poured into the duodenum during the first part of a meal, with consequent increase in the flow of pancreatic juice (Mellanby, '26). This observation alone is sufficient to establish the fact that the human gall bladder is a storage organ directly related to the process of digestion.

To see the effectiveness of this one has only to examine some of the extreme cases—e.g., case *C. H. S.* (fig. 4) in which the first phase discharged 16 cc. in ten minutes, or case

A. H. H. (fig. 6) in which the volume of the gall bladder was diminished 33 cc. in a phase lasting for twenty-five minutes. Furthermore, in young women with relatively small gall bladders this first period of contraction may empty the gall bladder of all but 1 cc. (fig. 8); even in larger ones, holding 27 cc. (e.g., *C. L. B.*, fig. 3), nine-tenths of the volume may be emptied by a first phase lasting only thirty minutes.²

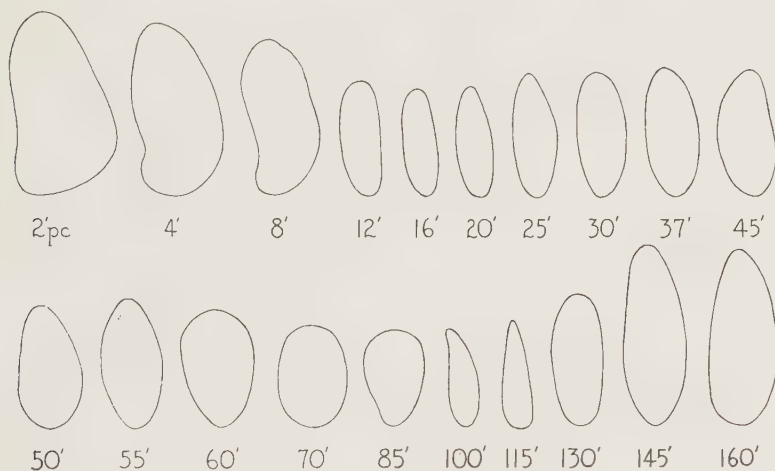


Fig. 7 Tracings of cholecystograms, illustrating a case in which the gall bladder emptied and filled twice, after a meal of five egg yolks and half a pint of cream (*C. H. S.*, fig. 8, *B*). During each period of filling the gall-bladder shadow became successively fainter, but so great was the original concentration of the dye that the shadow was still distinct after the second period of filling.

Succeeding phases of contraction

Following the termination of the first active phase, the gall bladder is generally quiescent for a short period varying from five to forty-five minutes, although in some instances the curve starts to descend again just before or as soon as it has reached the horizontal. Then comes the second phase of contraction, and this is frequently followed by several alternating periods of relaxation and contraction, until eventually

² For cholecystograms of these cases, see Boyden, '28 a (*C. H. S.*, fig. 1; *A. H. H.*, fig. 4; *C. L. B.*, fig. 2).

the gall bladder is emptied. This intermittency of action, which was first noted in the human gall bladder ('26 a), was subsequently observed by McMaster and Elman in the dog and by Whitaker in the cat. So that it may be presumed that this intermittency is characteristic of the mammalian gall bladder. In the studies of McMaster and Elman (the only ones in which the contraction rate of a laboratory animal has been recorded quantitatively) the intervals of contraction are much shorter than in human beings—six to thirteen minutes,

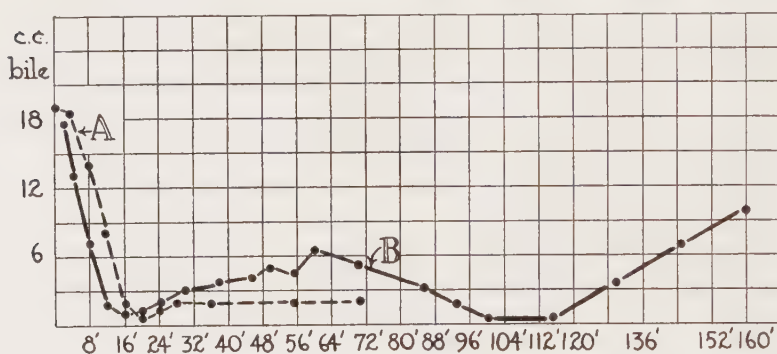


Fig. 8 Two graphs, showing, 1) the rapid initial emptying of small gall bladders after a meal of egg yolk and cream and, 2) their continued reaction to the presence of egg yolk in the stomach. In case *A* (*R. S.*, table 1) the gall bladder remained virtually empty for fifty minutes after having discharged all but 1 cc. of its contents. In case *B* (*C. H. S.*, table 1) it emptied and filled twice in less than three hours (fig. 7).

as compared with an average of thirty-two minutes for the first phase of contraction in man—and do not result (perhaps for this reason) in the complete emptying of the gall bladder (fig. 11).

Returning to a discussion of the later phases of contraction of the human gall bladder, it may be noted that those subsequent to the first are more or less irregular and not sharply defined. Moreover, they are less important, since one-half to three-quarters of the contents of the gall bladder has already been discharged. Also, since the organ is no longer distended and its smooth-muscle tunic less effective, the expulsion of

bile proceeds at a much slower pace, especially toward the last. Thus, while it sometimes happens that the gall bladder may be virtually emptied by the first or second phase of contraction, in the majority of cases it takes three or four phases to accomplish this result.

It is now appropriate to discuss the completeness with which the gall bladder empties. Some authors have felt the necessity of maintaining that the human gall bladder is never entirely purged of its contents, and have clung to this view as a means of explaining biliary stasis and the formation of calculi. As evidence, they have presented experiments on rabbits or dogs, in which the mechanism of emptying is admittedly less effective than in man. On the other hand, it is often claimed by others that the disappearance of a gall-bladder shadow after food is sufficient proof that the gall bladder has expelled all its contents. But this may indicate quite the opposite, the apparent disappearance being caused by sudden dilution of the iodized bile in the process of filling. There are only two ways of testing this problem—first, by directly examining the gall bladder at laparotomy and, second, by computing the volume of the smallest shadow which can be obtained before the cholecystogram disappears.³ Following the latter method, in the twenty-four cases recorded in this paper (tables 1 to 3), I have found that after a meal the lowest observed content of iodized bile ranges from 0.29 to 4.5 cc.—the average for twenty-four gall bladders being 1.77 cc. To express it somewhat differently, in nineteen out of twenty-four cases the volume of the last cholecystogram before the x-rays were discontinued or before the shadow disappeared was found to be less than $2\frac{1}{4}$ cc. and in six it was half a cubic centimeter or less. In the great majority of cases, therefore, there can be no question but that the human gall bladder is virtually emptied of its fluid contents after a meal of egg yolk and cream.

³ In cats I have repeatedly examined gall bladders of animals that have been fed egg yolk, and have found numerous cases in which there remained merely a trace of a viscid residue, or else less than a cubic centimeter of recently acquired dilute yellow bile, proving in either case that the gall bladder had been emptied of its fluid contents (compare also with Babkin, '28, p. 749).

TABLE 1

Discharge of bladder bile after egg yolk and cream in twelve young women

NAME	AGE	WEIGHT	VOLUME OF GALL BLADDER BEFORE MEAL ¹	PERCENTAGE OF BILE DISCHARGED AT			MINIMUM TIME REQUIRED TO EMPTY ALL BUT 3 CC.	VOLUME OF SMALLEST CHOLECYS- TOGRAM
				15' pc.	30' pc.	45' pc.		
	<i>years</i>	<i>pounds</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>minutes</i>	<i>cc.</i>
A. H. H.	24	160	43.54	54.9%	76.9%	78.0%	140	2.10
L. R. F.	21	119	42.80	55.5%	76.0%	84.1%	84	2.10
J. M. L.	22	130	41.47	34.7%	64.0%	68.2%	116	2.20
K. L. L.	22	128	34.53	57.8%	71.8%	82.7%	96	1.62
S. M. O.	24	122	32.61	50.2%	64.7%	67.4%	116	2.20
C. L. B.	29	115	24.50	72.2%	90.3%	91.5%	26	0.29
H. H. K.	23	190	21.25	71.2%	80.1%	88.4%	44	0.29
R. S.	34	121	19.17	15.4%	93.9%	93.9%	15	1.20
C. H. S.	29	110	17.71	91.7%	92.4%	92.4%	11	0.88
H. H. S.	28	155	15.79	17.0%	34.8%	56.0%	100	2.10
E. E.	17	106	14.86	40.0%	83.1%	89.0%	30	0.55
F. E. D.	18	120	14.32	19.0%	55.0%	69.4%	44	0.52
Average	24½	131.3	26.85	48.3%	73.6%	79.8%	68.5	1.33

¹ Body and fundus of gall bladder only.

TABLE 2

Discharge of bladder bile after egg yolk and cream in twelve young men

NAME	AGE	WEIGHT	VOLUME OF GALL BLADDER BEFORE MEAL ¹	PERCENTAGE OF BILE DISCHARGED AT			MINIMUM TIME REQUIRED TO EMPTY ALL BUT 3 CC.	VOLUME OF SMALLEST CHOLECYS- TOGRAM
				15' pc.	30' pc.	45' pc.		
	<i>years</i>	<i>pounds</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>minutes</i>	<i>cc.</i>
H. M. T.	24	169	50.78	0	42.8%	53.1%	216	0.30
I. D.	18	145	42.50	34.2%	48.1%	59.7%	184	3.24
N. A. C.	25	141	35.10	25.2%	45.1%	56.3%	145 (4.5 cc.)	4.50
N. B. B.	27	165	32.76	65.0%	81.0%	88.7%	50	2.00
L. O. M.	26	175	31.86	68.1%	85.2%	90.6%	48	0.50
C. C. C.	26	160	30.99	31.4%	57.1%	62.5%	225 (3.83 cc.)	3.83
E. F. S.	29	142	26.57	14.2%	32.2%	45.0%	114	0.74
V. M.	28	142	25.83	38.3%	48.6%	54.2%	135 (4.3 cc.)	4.30
R. L. W.	24	112	25.24	49.1%	55.5%	67.9%	105	1.18
B. J. A.	31	154	24.79	16.5%	62.4%	76.7%	84	1.03
C. E. F.	26	150	21.98	0.5%	13.3%	41.6%	136	1.90
F. K. H.	19	155	21.24	50.6%	56.9%	71.6%	100	3.00
Average	25½	151.1	30.8	32.8%	52.3%	64.0%	128.5	2.21

¹ Body and fundus of gall bladder only.

The rate of emptying

In examining a large number of x-ray series in which the attempt has been made to keep every factor constant, one is astonished at the range of variation exhibited by the human gall bladder. That this is not due to the fickleness of any given individual is shown by the constancy with which an individual reacts when subjected twice to the same stimulus. In figure 6, for instance, are two gall-bladder series from the same person taken two months apart, the only difference being that in one case the x-rays were made with the patient reclining and

TABLE 3

Sex differences in emptying of human gall bladder (based on tables 1 and 2)

	AGE	WEIGHT	VOLUME OF GALL BLADDER BEFORE MEAL	PERCENTAGE OF BILE DISCHARGED AT			MINIMUM TIME REQUIRED TO EMPTY ALL BUT 3 CC. OF BILE	VOLUME OF SMALLEST CHOLECYSTOGRAM
				15' pc.	30' pc.	45' pc.		
	<i>years</i>	<i>pounds</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>cc.</i>	<i>minutes</i>	<i>cc.</i>
Average for 12 women	24½	131.3	26.8	48.3%	73.6%	79.8%	68.5	1.33
Average for 12 men	25½	151.1	30.8	32.8	52.3	64.0%	128.5	2.21
Average for 24 cases	24½	141.1	28.8	40.5%	62.9%	71.9%	98.5	1.77

in the other case with the patient erect. Yet the rate of emptying in the two series is almost the same. When, however, different individuals are subjected to the same stimulus, no two react quite alike, the extremes ranging from gall bladders which empty in sixteen to twenty minutes (fig. 8) to those which take four hours and a half (*H. M. T.*, Boyden, '26 b, p. 235).

That the length of time required to empty a gall bladder is partly due to the initial size of the reservoir may be gathered from an examination of tables 1 and 2 in which twenty-four gall bladders are arranged according to their volume. In general, the larger ones on the upper half of each table take

longer to empty than the smaller ones in the lower half. But the rate of emptying is not entirely a function of size, as may be ascertained by comparing the angle of the descent of the curves in large and small gall bladders (fig. 3). Another factor seems to be the sex difference, to which the writer has called attention in a preliminary publication ('27 a). Since this report was published, ten more cases have been added, making a total of twenty-four individuals (tables 1 and 2), with the result that the comparison is even more striking than had been anticipated from the preliminary study. The detailed summary is given in table 3, from which it appears that on the average the expulsion of bladder bile is much more effective in the female than in the male. The extent of the difference may be gauged by the fact that, forty-five minutes after a meal, the male gall bladder has not discharged as much bile relatively as the female gall bladder has expelled in the first thirty minutes. Measured in another way, it takes the former nearly twice as long as the latter to reduce its volume to an arbitrary content of 3 cc.

In attempting to determine whether the rate at which food was discharged from the stomach and the rate of intestinal peristalsis were factors underlying these individual and sex differences, the writer and Doctor Birch ('27) made a comparative study of the emptying time of the gall bladder and the motility of the gastro-intestinal tract in six individuals, but were unable to establish any specific relation of cause and effect. Thus an individual with moderately slow evacuation of the stomach (six hours) and a hypomotility of the gut tract induced by chronic constipation (120 hours) was rated first among twelve men, from the standpoint of the emptying of the gall bladder (*L. O. M.*, fig. 3). Conversely, a young woman with average motility of the intestine (seventy-two hours) and a hypermotile stomach (four hours) was among the slowest of twelve associates in discharging bladder bile (*H. H. S.*, fig. 3). Nor could any correlation be made between the varying acidity of the stomach in different individuals and the motility of the gall bladder.

Whether a much larger series would have shown a general correlation between the motility of the gut tract and that of the biliary tract is at present a matter of conjecture. The same month that these data were published, an English investigator, Dr. Francis Davies, arrived at the conclusion that there is a correlation between the rate of emptying of the gall bladder and bodily habitus—that the gall bladder empties faster in hypersthenic and sthenic types than in hyposthenic and asthenic individuals. Since Mills had previously established a correlation between gastric motility and bodily habitus, Davies assumed that “a definite correlation is thus indicated between gastric motility and the rate of emptying of the gall bladder.” But it seems that he measured the extent of reduction in the size of the gall-bladder shadow by only one x-ray, which was taken two hours after a fatty meal. From the writer’s detailed study of twenty-four cases it would appear that very little dependence can be placed on this single observation, for the gall bladder might have emptied twice in two hours (fig. 8), or have emptied early and partially filled again, or have maintained a volume which at two hours had just been reached by a much slower vesicle. Also it is not clear from the author’s description whether or not the fatty meals which were given the students were exactly alike in each case. Therefore, while appreciating certain phases of this work, the writer believes with Graham ('28, p. 283) that any correlation between the rate of emptying of the gall bladder and bodily habitus has yet to be demonstrated.

Nevertheless, it is very difficult to find a satisfactory explanation for either individual or sex differences in the emptying time of the gall bladder. There are morphological differences, of course, which may affect the flow of bile, such as the varying number and disposition of the valves in the cystic duct or the amount and arrangement of the muscle tissue constituting the sphincter papillae, which, according to Giordano and Mann ('27), varies considerably in different species and in individuals of the same species. But at present there are no data which encourage the belief that sex differences in the

emptying time of the gall bladder can be referred to morphological differentiation. Nor at present does there seem to be any underlying physiological explanation, for apparently no other sex differences in the physiology of the digestive tract have ever been noted.

EVIDENCE SUGGESTING REGULATORY ACTION BY THE SPHINCTER
MECHANISM AT THE OUTLET OF THE COMMON DUCT IN MAN

In any discussion of the mechanism of the flow of bile, two factors are of prime importance: the force which expels the bile from the gall bladder and the method by which the flow of bile into the duodenum is regulated. Since the evidence now seems conclusive that the musculature of the gall bladder is responsible for the expulsion of the contents of the organ, it is possible for the flow of bile to be regulated in only two ways—either exclusively through the activation or inhibition of this expelling musculature or by the addition to this of a supplementary mechanism which controls the flow of bile at the outlet of the common duct. (Luetkens' theory of control by a collum-cysticus sphincter does not seem to be supported by any convincing evidence. Boyden, '28 a, p. 40.)

That there is such a dual regulation of the bile flow in certain laboratory animals is indicated by the following experiments. When the common duct was severed in a cat and the animal was fed egg yolk, the gall bladder responded by successive phases of contraction and expulsion of bile, even though it was no longer connected with the intestine (Boyden and Birch, '27). Therefore, control under the conditions of this experiment was exerted solely by something which activated the musculature of the gall bladder intermittently—presumably, in this case, the hormone cholecystikinin, discovered by Ivy ('27 a). In another cat it was fortuitously observed that the flow of bile after egg yolk was modified by an antiperistaltic wave which prevented the expulsion of bile into the intestine, as a result of which the contracting gall bladder injected its contents into the hepatic-duct system ('26 b, pl. 1). These two experiments with cats thus reveal

the existence of a regulation of the flow of bile at both ends of the system. What evidence is there that such a dual control exists in man?⁴

⁴ The experiments just cited raise the issue as to whether the regulation of the flow of bile at the duodenal orifice is due primarily to the activity of the intestinal musculature or to the action of an intrinsic sphincter around this end of the common duct. In the best article that has appeared on the subject, Giordano and Mann ('27) write as follows:

That peristalsis, variation in tone and spasm of the duodenal wall would influence the discharge of bile has never been questioned. It is difficult to prove that such factors are the only or prime ones controlling the passage of bile into the duodenum, and there are several facts that militate against such a view, making it appear that a true sphincter is essential.

Among these facts it is noted that, in animals with a gall bladder, the bile is discharged from the outlet of the common duct in intermittent spurts without any discernible peristalsis; but that if the papillary orifice is gently irritated with a sponge, the outflow of bile may be inhibited for a considerable period and then may be discharged with a large spurt without producing any discernible change in the duodenal wall. Yet if a rat or a pocket gopher, a species normally without a gall bladder, is examined in the same manner (i.e., through a duodenal window) the flow is never intermittent nor is the bile discharged in spurts; and sponging the opening of the duct does not inhibit the discharge. These observations definitely prove that there is a difference in the discharge of bile in the two groups of animals and that this is due to the presence of a sphincter mechanism in one and not in the other.

Giordano and Mann have also met such experiments as those of Burget ('26) and Berg and Jobling ('27) by the argument that, if the transplanted common duct is sutured sufficiently tight to prevent leakage of the duodenal contents, a certain degree of obstruction of the duct will occur which would assist the gall bladder in filling and would prevent too rapid a discharge. To this argument I would add another, that one is not yet justified in stating that "normal cholecystograms may be obtained in dogs after division and transplantation of the common duct," for has anyone yet established the 'normal' emptying time of the gall bladder in dogs? And even if such data were available, it would be necessary to have previously ascertained the normal emptying time of the dog used in the experiment, so varied is the individual response to the ingestion of food. Furthermore, Higgins and Mann ('27) were able to dissect the intramural portion of the common duct in dogs and to locate an intrinsic sphincter which constricted the lumen of the duct when stimulated with an induction current.

In man there is the clinical observation that cholecystectomy is followed by dilatation of the common duct—an after-effect which can be fully explained only by the presence of a sphincter mechanism at the orifice of the duct. Finally, there is the long list of investigators from the days of Gage and Oddi (see Giordano and Mann) who have noted the anatomical occurrence of such a sphincter in man and certain laboratory animals. One of the most interesting of these accounts is the latest one (Matsuno, '23), in which it is noted that the muscle fibers of the sphincter in man are smaller than those of the adjacent musculature, but constitute a band 5 to 10 mm. in width.

The case of an hour-glass gall bladder

In the course of accumulating twenty-five x-ray series illustrating the reaction of the human gall bladder to a meal of egg yolk, it was found that the gall bladder responded by emptying most of its contents in all but one case. This was

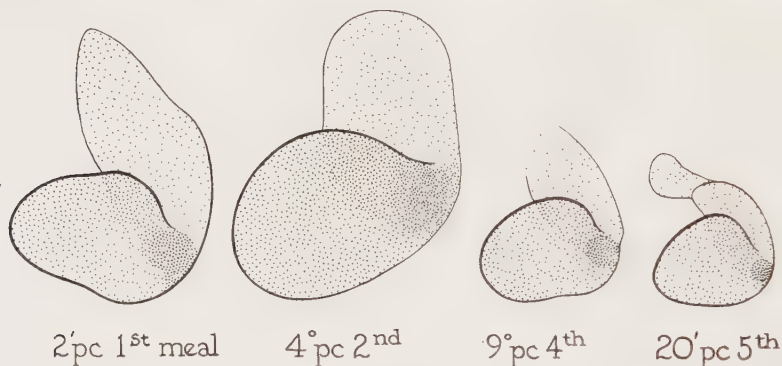


Fig. 9 Tracings of selected cholecystograms, to show the changing shapes of an hour-glass gall bladder (case *D. W. R.*; compare fig. 10 and pl. 1).

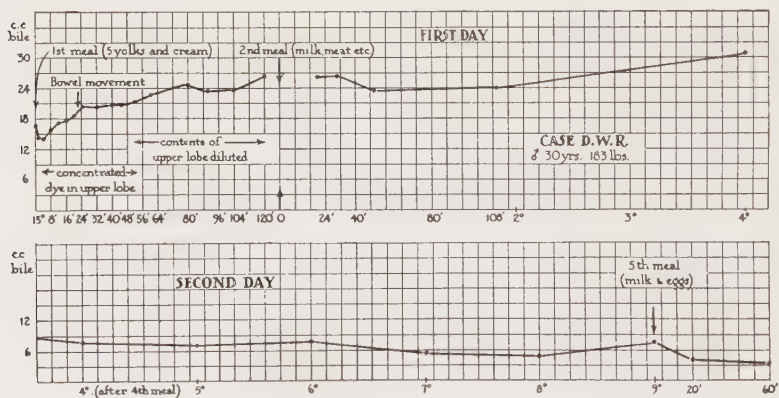


Fig. 10 Chart illustrating the change in volume of the lower half of the hour-glass gall bladder shown in figure 9 and plate 1 during the two days in which this individual was under observation. During the first day the gall bladder continued to fill (indicating a closed sphincter), notwithstanding the fact that two meals were eaten and that active peristalsis was evident (as suggested by the occurrence of a bowel movement). Yet, during the first fifty-two minutes after egg yolk, the gall bladder was under contraction as indicated by the concentrated dye forced into the upper lobe.

a strong, muscular student, whose gall bladder was separated into two big lobes by a deep incisure (fig. 9), the connecting aperture being so small as to greatly retard diffusion of the bile from one chamber to the other. When this individual was given a meal of egg yolk, the gall bladder failed to empty (fig. 10). Some two hours later, a second meal, of different character, was given, but without inducing more than a temporary response. But after the fourth meal, on the second day, the volume of the gall bladder was found to be markedly reduced, whereupon a meal of egg yolk was given which this time was successful in further emptying the gall bladder. The question at once arose as to why the gall bladder of this individual emptied on the second day, but not on the first.

The answer to this question was made possible by the smallness of the orifice connecting the two lobes, which prevented rapid diffusion of the fluid from one lobe to the other and thus enabled us to tell when it was under contraction and when it was relaxed. Before the first meal, the dye in the lower lobe was more concentrated than in the upper (fig. 20). As soon as the egg yolk was taken, the fundus contracted and forced the more concentrated dye of the lower lobe into the upper lobe and held it there for fifty-two minutes (fig. 21). Then suddenly the dye in the upper lobe became diluted (fig. 22), from which it must be assumed that the gall bladder relaxed and admitted a flow of dilute hepatic bile. But during the first fifty-two minutes when the gall bladder was under contraction, its volume after the discharge of the first few minutes was slowly increasing (fig. 10). This can be accounted for only by the existence of a spastic closure of the outlet of the common duct which prevented the emptying of the gall bladder even though it continued to contract. The fact that this gall bladder emptied after egg yolk on the second day, however, showed that it was not a diseased gall bladder and that the state of shock was probably induced by the presence of the iodine salts. This was verified by a second experiment on this same individual a few months later, when egg yolk was introduced directly into the duodenum by a Rehfuß

tube, after which the sphincter still failed to open, although the gall bladder contracted.⁵

The two-minute pause

A second line of evidence, suggesting a dual control of the flow of bile, is offered by the existence of the peculiar irregu-

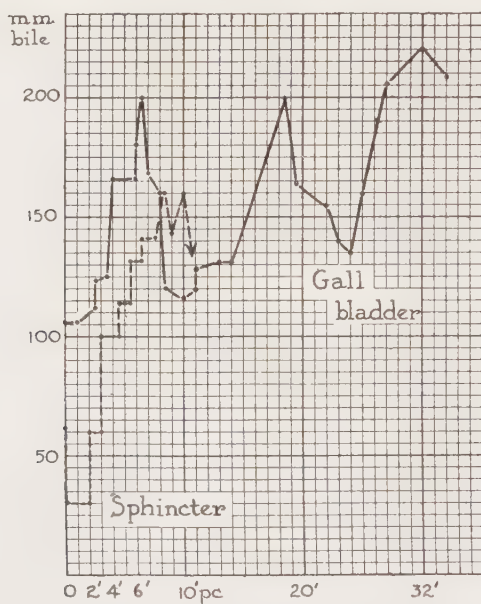


Fig. 11 Chart showing the reaction of the biliary tract to the ingestion of food in the dog. Solid line, changes in intravesicular pressure of the gall bladder, illustrating three intermittent phases of contraction (after McMaster and Elman, '26, fig. 6); dash line, changes in the resistance offered by the duodenal sphincter (after Elman and McMaster, '26, fig. 3). 50, 100, etc., resistance measured in millimeters of bile; 2', 4', etc., minutes after food.

⁵ It is interesting to compare this condition of temporary shock with the permanent changes which overtake the sphincter. Giordano and Mann have reported that "certain specimens examined at necropsy suggest the possibility that the sphincteric mechanism can be thrown into spasm by pathological conditions in the gastro-intestinal tract and adjacent organs." As basis for this they have published dissections of the duodenum in some cases of cholecystitis and peptic ulcer which show "definite hypertrophy of the muscle bundles around the common duct, not only in the intramural portions of the duct but sometimes, also, extending a considerable distance up the duct from the duodenal wall. In some of these instances pancreatitis was also found."

larity in the curve of the first phase of contraction, which has been previously designated as the 'two-minute pause' (fig. 3). When it became clear that this was a fairly constant feature, the question arose as to why the flow of bladder bile should be temporarily checked at the end of the first two minutes. The matter was further complicated by the observation that the shape of the gall bladder during the 'two-minute pause' did not indicate relaxation, but rather continued contraction (compare 2' and 4' *pc*, fig. 2). Coupled with this was the interesting fact that the reduction of gall-bladder volume was greater in the first two minutes than any subsequent two minutes. The implication, therefore, was that the irregularity in the curve was due to the action of the duodenal sphincter, and not to the gall bladder: that, following the ingestion of food, the orifice of the common duct was suddenly opened, permitting the gall bladder to fill the extra-hepatic-duct system without hindrance;⁶ and that, two minutes later, renewed closure of the orifice checked the flow of bile from the gall bladder temporarily, even though the latter continued to contract.

Additional evidence for this view is supplied by the work of McMaster and Elman on the dog. By an ingenious method of triple intubation they were able to record simultaneously three events occurring after meals, namely, the rise in pressure within the gall bladder, the flow of bile from the liver, and the resistance offered by the sphincter at the end of the common duct. To better illustrate the relation of their findings to my own observations I have taken the liberty of combining two of their figures into one diagram (fig. 11), the upper curve of which records the response of the gall bladder and the lower curve the reaction of the sphincter.

This chart shows that, simultaneously with the ingestion of food, there is a drop in the resistance of the sphincter. Approximately a minute later, the gall bladder begins to con-

⁶ Recent work of Doctor Birch and the writer in aspirating the contents of the duodenum has shown that it takes from eight to fourteen minutes after egg yolk to recover the first bladder bile from the duodenum.

tract. As a third step in the sequence of events the resistance offered by the sphincter suddenly increases, beginning two minutes after the ingestion of food, and continuing until it is greater than the force exerted by the gall bladder.

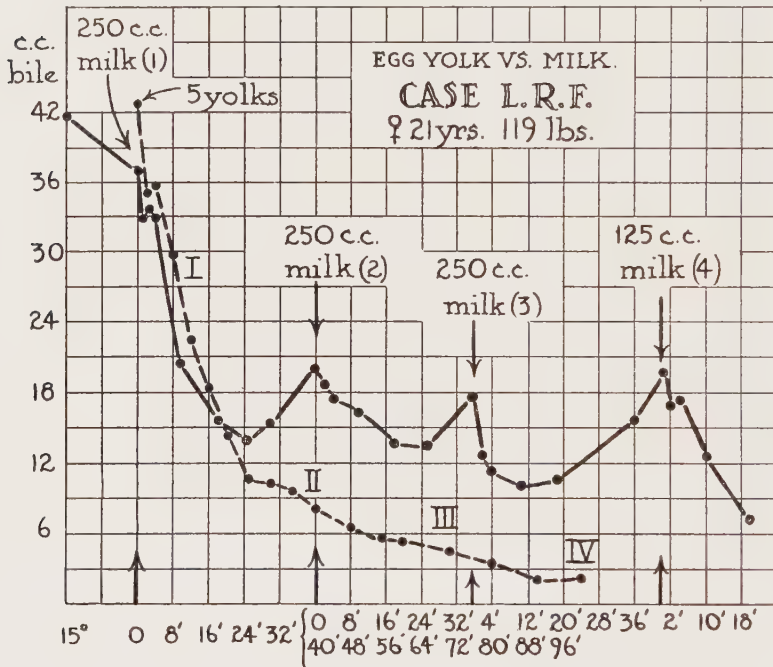


Fig. 12 Contraction curves from same individual, taken several months apart. The first series (after yolk and cream) resulted in four successive phases of contraction, the first of which is the most effective. The second series (after four successive glasses of milk) shows that milk causes only one phase of contraction and suggests that the phases of contraction after egg yolk are due to repeated spurts of yolk-laden chyme from the stomach (compare fig. 13, series 2).

The striking agreement of these data with the observations recorded by the writer seems to afford the most satisfactory basis for interpreting the contraction curves of the human gall bladder. Thus interpreted, the first phase of contraction may be described as follows: As soon as egg yolk is ingested, the orifice of the common duct opens, releasing the pressure in the biliary-duct system. Within less than a minute, the

gall bladder begins to contract (perhaps at first as a result of the stimulation of the sensory nerve endings in its wall induced by this sudden release of pressure), and rapidly fills the duct system.⁷ This has been defined as the period of 'initial response.' In another minute, the flow of bile from the gall bladder is checked through the increased resistance offered by the sphincter (the interval of the 'two-minute pause'). But under increased stimulation, following the absorption of yolk, the initial resistance of the sphincter is either modified or overcome by the increased power of the gall bladder, whereupon the latter continues to pour out bile until the mounting resistance of the sphincter ends the first phase of emptying.

Subsequent spurts of egg yolk from the stomach would then renew the sequence of events, thus accounting for subsequent phases of contraction. This can be tested in several ways. When, for instance, the number of phases of contraction have been determined after egg yolk in a given case (as *I* to *IV*, fig. 12), these phases can be reproduced by having the patient drink at appropriate intervals just as many glasses of milk. Since the effective portion of milk is not retained in the stomach ('26 c), the single contraction induced by each glass of milk will be followed by a filling of the gall bladder. In the case in question, if the successive glasses of milk had been given at thirty- instead of forty-minute intervals, the continuous descent of the yolk curve would have been more nearly duplicated. But at that time a longer interval was allowed, since it was suspected that each phase of contraction was followed by a refractory period. The first feeding of egg yolk in figure 13 (series 3) would also seem to support that idea. But experience with a larger number of cases in which substances have been injected into the duodenum (Boyden and Birch, '28) has led me to doubt the existence of a refractory period for the gall bladder.

⁷ For experimental evidence of the presence of sensory nerve endings in the wall of the gall bladder, see Schrager and Ivy, '28.

A supplementary case is shown in figure 13 (series 2), where small amounts of egg yolk were injected directly into the duodenum at intervals. After each injection the gall bladder responded by immediate contraction. This series emphasizes three facts. First, that the initial effect is greater

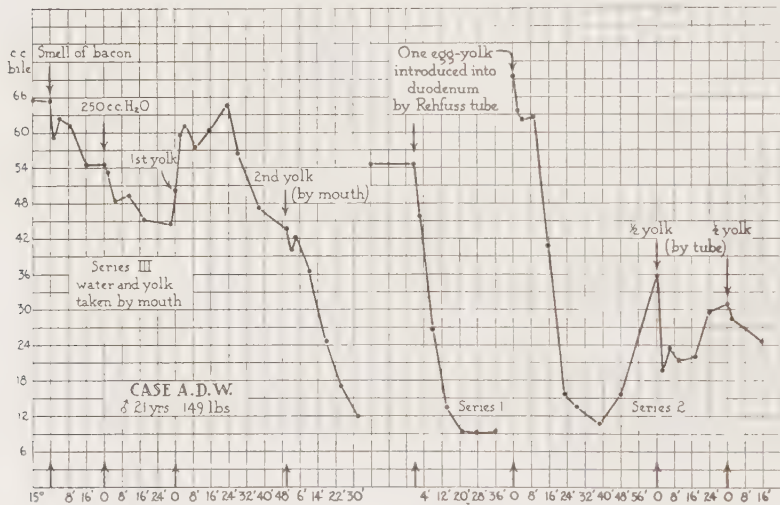


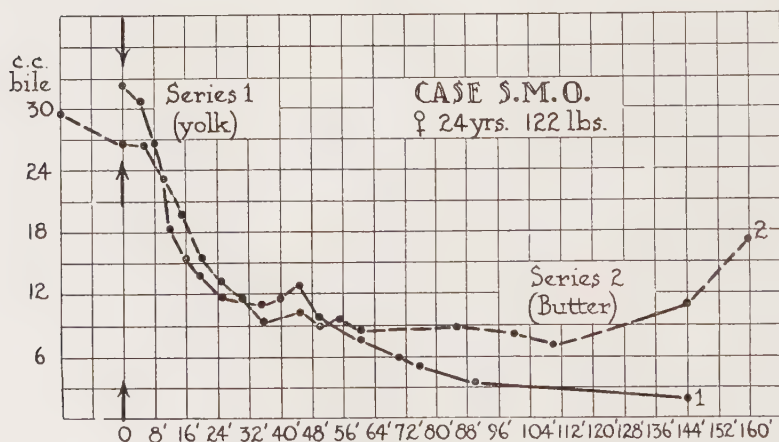
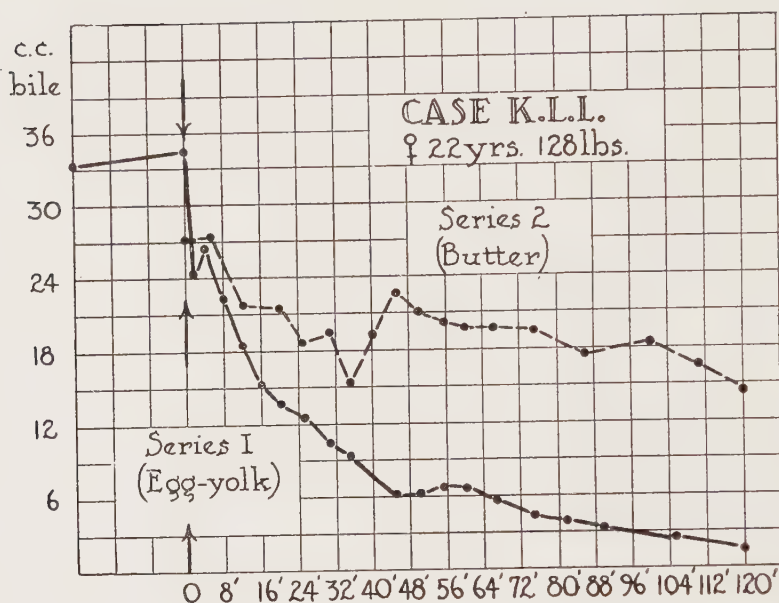
Fig. 13 Three contraction curves from the same individual taken several months apart, to illustrate the greater effectiveness of egg yolk when injected into the duodenum (series 1) than when given by mouth (series 3). Series 2 shows that one yolk placed in the duodenum causes as pronounced a first phase of contraction as five yolks introduced into the stomach. On the other hand, each successive small quantity of yolk placed in the duodenum causes only one phase of contraction. Therefore, if the gall bladder is to be emptied completely, sufficient yolk must be taken by mouth to insure repeated spurts of yolk-laden chyme (compare fig. 12). *Series 2*, from unpublished data by Boyden and Birch.

when egg yolk is introduced directly into the duodenum than when taken by mouth (compare *series 1* and *3*, figs. 13 and 17); secondly, that a food stimulus is most effective when applied to a distended gall bladder, probably since the smooth muscle responds more vigorously when somewhat stretched—an analogy for which may be found in the action of the urinary bladder; and, thirdly, that the mechanism of contraction is a

trigger mechanism set off by a variety of fatty foods and that the entrance into the intestine of each dose of an effective food results in a single phase of emptying which is immediately followed by filling. Incidentally, it shows that one egg yolk by mouth is not sufficient, but that enough must be eaten to insure sufficient spurts of yolk-laden chyme to induce the number of successive phases required to empty the gall bladder. Also it emphasizes the importance of selecting the right kind of fatty food if the gall bladder is to be completely emptied. Butter, for instance, is not nearly so effective (figs. 14 and 15). It is of interest, however, as being the food which was used by Whitaker and his associates—not knowing of the writer's demonstration two years earlier (1923) of the complete emptying of the mammalian gall bladder after egg yolk—in first demonstrating marked reduction in volume of the human gall bladder after food (compare Whitaker, '26, p. 411).

The reaction to odors of food, to the drinking of water, and to the distention of the duodenum with air

Odors of food. The third line of evidence which suggests regulation of bile flow by a sphincter mechanism around the outlet of the common duct is the reaction of the gall bladder to substances other than food. The very clear demonstration by McMaster and Elman of the effect of odors upon the sphincter of dogs induced me to try this form of stimulation upon human beings. Accordingly, a large number of patients were submitted to an experiment which consisted of suddenly placing a dish of hot fried bacon under their noses as they lay reclining on the x-ray table. Since they had received no intimation of this procedure nor had eaten anything for fifteen hours, they all developed a ravenous appetite. During the ten minutes in which they continued to smell the bacon, x-rays were taken at short intervals whereupon it was found that eight out of eleven cases responded by an average reduction of 6 cc. in the volume of the gall bladder, while the remaining three were negative or showed an increase in volume (cases I,



Figs. 14 and 15 Two charts, consisting of two contraction curves from each of two individuals, showing the comparative response of the human gall bladder to egg yolk and to butter. *Series 1*, in each case, records the reaction to five egg yolks and half a pint of cream. *Series 2* shows the response to varying amounts of butter— $\frac{3}{8}$ of a pound on six plain crackers (*K. L. L.*); and $\frac{1}{8}$ of a pound on seven crackers (*S. M. O.*).

and IX, fig. 16; and fig. 1). When the eight positive cases were analyzed, it was found that an average of 4.2 cc. of bile was discharged by four of these cases in the first two minutes of smelling (cases III, VIII, and X, fig. 16, and series 3, fig. 13). This corresponds to the amount discharged during the period of 'initial response' after egg yolk and presumably is due to the opening of the sphincter, if we may judge from the experiments on dogs. In two of these four cases, however (no. X and series 3, fig. 13), the initial discharge was followed by a 'two-minute' pause, after which there was a second discharge lasting six to twelve minutes, which averaged more than the first, or 7 cc. In the other four positive cases there was no initial response, but after a delay of four to six minutes there occurred a discharge which averaged 8 cc. (cases IV, V, and XI, fig. 16). The fact that in five instances a considerable expulsion of bile occurred four to six minutes after odors were first smelled suggests that in these cases we are dealing with a second factor—perhaps a second contraction of the gall bladder induced by the entrance of hydrochloric acid into the intestine, following psychic stimulation of the gastric glands. More will be said about this later.

Water. Similarly, the drinking of a glass of cold water resulted in two kinds of reaction. In the first group of cases the response consisted of an initial discharge of bile, such as might have been induced by the opening of the sphincter. In the other group the initial flow was supplemented by a subsequent discharge or else there was a delayed discharge. But, in general, the effect of water was more consistent than the smell of food, causing an expulsion of bile in each of the fourteen cases which were observed; also the discharge of bile was twice as great, the average amounting to $8\frac{1}{3}$ cc. In three of the cases (nos. I and XII, fig. 16; series 3, fig. 17) there was a delayed emptying of the gall bladder following an initial rise in volume. In six others there was an initial fall (averaging $6\frac{1}{4}$ cc.) during the first two to twelve minutes (cases III, IV, VI, VIII, X, and XI, fig. 16). In the remaining five patients there was an initial discharge lasting two to six min-

utes, a pause of two to fifteen minutes, and a second discharge from four to thirty-five minutes (cases *II*, *V*, *VII*, and *IX*,

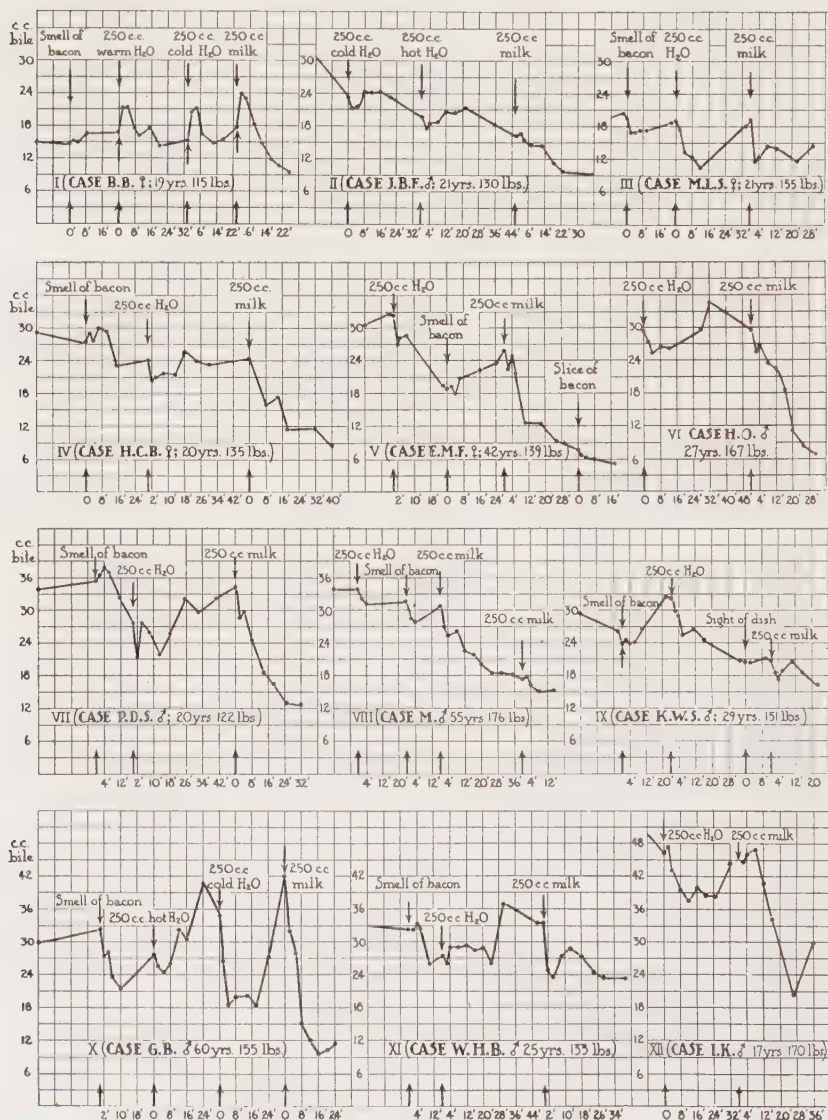


Fig. 16 Twelve cases showing the reaction of the human gall bladder to the smell of bacon or the drinking of a glass of water or a glass of milk.

fig. 16; and *series 3*, fig. 13). The contraction curve of this last group, therefore, resembles the curve obtained by the ingestion of food, in that it consists of two discharges with an intervening pause—one due to the opening of the sphincter and the other to some other factor. In the case of food,

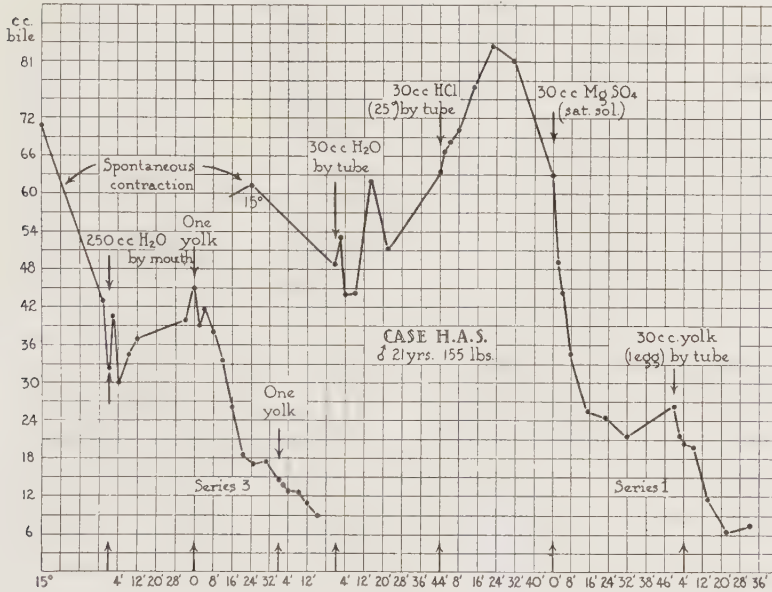


Fig. 17 Two contraction curves from the same individual comparing the effect of a glass of water taken by mouth with a small amount of water introduced by tube into the duodenum. The effect upon this individual in each case is the same: the first response causing inhibition of the gall bladder, followed by moderate discharge of bile. *Series 3* also suggests that egg yolks swallowed one by one and properly spaced would be as effective in emptying the gall bladder as a large number eaten all at once. Note spontaneous contraction in *series 3*.

the second or main discharge is presumably caused by the absorption of food and the production of the hormone cholecystokinin (Ivy, '27 a). In the case of water, the second discharge might be accounted for by the absorption of water or by the action of the gastric juice which is washed into the duodenum. Attempts to separate the action of water and HCl by injecting these substances directly into the duodenum

are still in progress. The matter is complicated by the fact that, in some cases, injection of water into the duodenum induces a spurt of chyme from the stomach (unpublished data, Boyden and Birch). A similar problem is offered by the next set of experiments.⁸

Distention of the duodenum with air. In the hope of answering the question whether the opening of the sphincter and the first discharge of bile from the gall bladder might not be due merely to the mechanical distention of the duodenum, air was injected into four patients by means of a Rehfuß tube (Boyden and Saunders, '28, fig. 1). Those portions of the curves recording the effect of air are reproduced in figure 18. In two of the cases (*E. I.* and *M. A.*) the injection of air produced almost exactly the same initial discharge of bile as occurs during the first two minutes after egg yolk (an average of $4\frac{2}{3}$ cc. in these two cases), and therefore would seem to be due to reflex relaxation of the sphincter caused by mechanical distention of the bowel. In the third case (*A. R.*), the period of 'initial response' was followed by a 'two-minute

⁸ The action of HCl on the musculature of the gut tract is one of the most difficult problems in physiology, especially its action on the pyloric sphincter (compare Alvarez, '28, pp. 178-195). Its influence upon the flow of bile needs equally careful investigation. According to Babkin ('28, p. 687), Claude Bernard was the first to show that a drop of HCl placed on the intestinal opening of the common duct resulted in a flow of bile. In 1915, Okada reported that, when 0.4 per cent HCl was placed in the lower part of the duodenum, a rapid outpouring of the bile was observed after two or three minutes. The same happened when all the hepatic ducts were tied, so that Okada rightly concluded that HCl caused expulsion of bile from the biliary-duct system. In 1926, Elman and McMaster showed that N/10 HCl, given by stomach tube to dogs, caused reflex relaxation of the sphincter; and a year later, Ivy demonstrated by an ingenious cross-circulation experiment that HCl injected into the duodenum of one dog caused a contraction of both gall bladders, presumably by means of a hormone traveling in the blood stream. So that, apparently, both sphincter and gall bladder are affected by HCl. But Doctor Birch and I have found that when HCl is injected into the human duodenum—in amounts occurring in the normal stomach—it is not so constant in its effect as a glass of water taken by mouth and causes nowhere near so great a discharge of bile as egg yolk or such saline cathartics as MgSO_4 , MgCl_2 , and Na_2SO_4 . It may be, however, that it is a contributory factor in such minor discharges of bile as are induced by the smelling of food and the drinking of water—a problem which is under further investigation.

pause' and a second period of discharge. This renews again the problem raised by certain of the cases recording the effect

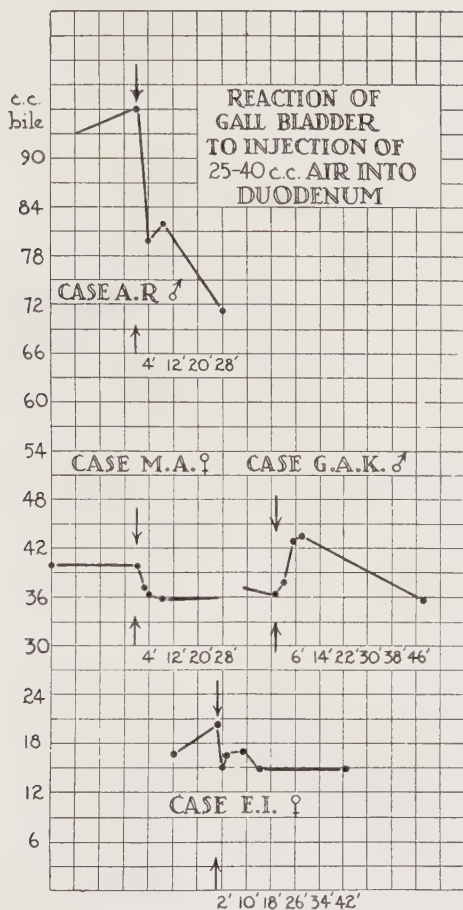


Fig. 18 Portions of four contraction curves taken from a chart already published (Boyden and Saunders, '28, fig. 1), to show the effect on the gall bladder of the injection of air into the duodenum. In three out of four cases it caused contraction; in the fourth case (*G. A. K.*), sudden filling of the gall bladder.

of smelling food and of drinking a glass of water—cases in which all three portions of the first phase of contraction (following egg yolk) were reproduced, the only difference being that the principal period of discharge is much less,

quantitatively, than after egg yolk or milk or such salts as MgSO_4 . What could cause this second or principal discharge in the case of air (*A. R.*, fig. 18)? Possibly the injection of air into the duodenum causes a spurt of chyme to be injected from the stomach, which renews the contraction through the temporary action of hydrochloric acid. Or some physiologists would suggest that peristalsis of the bowel induced by distention would account for the second discharge. But it has been shown that contraction of the gall bladder can be induced after that organ has been disconnected from the intestine (Boyden and Birch, '27). Also our experience with saline cathartics ('28) tends to minimize the effects of peristalsis in regulating the flow of bile, since of all the hydragogue cathartics which distend the bowel and supposedly induce peristalsis, only a few cause evacuation of the gall bladder. We are therefore inclined to postulate the existence of some unrecognized factor, especially since no satisfactory explanation has yet been given for the marked spontaneous contractions which sometimes occur before meals (*series 3*, fig. 17).

THE THREE TYPES OF RESPONSE

In summarizing the data contained in this paper it has seemed to the writer that these cases fall into three main groups. The first of these is a type in which a given stimulus simultaneously induces sudden closure of the sphincter and relaxation of the gall bladder. Such a one is shown in figure 16 (*B. B.*), in which the drinking of 250 cc. water and later of 250 cc. milk resulted in initial filling of the gall bladder instead of in partial emptying. So sudden and pronounced was the change—from a volume of 17.5 to 24 cc. in two minutes—that it seemed best interpreted as a reflex inhibition of the gall bladder. That there was a sudden relaxation of the tunica muscularis was apparent from the remarkable change in shape of the cholecystograms (Boyden and Parmacek, '28, p. 464). Some time later, the same patient was given much smaller amounts of water and of milk (30 cc.)—this time injected into the duodenum by Rehfuess tube—and again there

was the same reaction, namely, a sudden relaxation and filling of the gall bladder (Boyden and Birch, unpublished data). A second instance of this sort is the case shown in figure 18 (*G. A. K.*) in which injection of air into the duodenum caused sudden closure of the sphincter and immediate filling of the gall bladder. A third illustration is supplied by one of the series in figure 4 (*I. D.*) in which the same effect was produced by drinking a glass of yolk and cream—the gall bladder dilating 6 cc. in volume in the first two minutes. These instances are relatively rare and result in merely a temporary delay in emptying of the gall bladder. To see if perchance such individuals were differently orientated as regards the sympathetic nervous system, all the cases in figure 16 as well as some others were given the Goetsch test. The first cases (Boyden and Parmacek) seemed to indicate that inhibitory reactions of the gall bladder might be characteristic of vagotonic individuals, but a larger number of cases has caused us to abandon this theory. So that all we can say about these cases at present is that they illustrate one extreme in the response of the biliary mechanism, namely, simultaneous closure of the sphincter and relaxation of the gall bladder.

At the other extreme—e.g., case X, figure 16—stands that type of response in which the smell of food or the ingestion of food results in simultaneous relaxation of the sphincter and contraction of the gall bladder. This is the type which is the most effective and produces the greatest flow of bile in the shortest time.

The third type is a sluggish type, well represented by case II, figure 16, in which the first response to water is a momentary emptying followed by a slow filling and then by slow emptying, as if in this individual there was a hypertonic sphincter, which closed after the first stimulus had opened it and then only gradually relaxed. These cases are fairly common (*N. A. C.*, *H. H. S.*, *F. E. D.*, and *E. E.*, fig. 4) and represent inefficient, slow-discharging gall bladders. That the difficulty in such a type is with the sphincter, and not with the gall bladder, is indicated by the speed with which the gall

bladder discharges during the first two minutes when it is merely filling the common duct (*N. A. C.*, fig. 4), and not forcing bile into the duodenum. Finally, it may be stated that the importance of this type depends on the possibility that a hypertonic sphincter may in time produce a condition of physiological stasis, with the consequent formation of calculi.

That the gall bladder is a reservoir and discharges its accumulated contents by the contraction of its intrinsic musculature is no longer a matter of debate; but there remains a yet greater problem—the regulation of the flow of bile into the intestine.

CONCLUSIONS

The observations recorded in this paper are based on fifty successful x-ray series (each consisting of some twenty to twenty-five cholecystograms) which have been accumulated during the last two years. These do not include some twenty additional series (made in conjunction with Dr. C. L. Birch) in which the human gall bladder has been x-rayed at intervals following the introduction of various substances into the duodenum. The present study is an attempt to analyze the reaction of the evacuating mechanism of the gall bladder to the ingestion of food, a summary of which is given below:

1. Before a meal the gall bladder is not quiescent, but is either slowly filling or contracting. Some of these spontaneous contractions are almost as great as those induced by food.

2. The emptying of the human gall bladder after a meal of egg yolk and cream is intermittent in character, consisting of from one to five phases of contraction, depending somewhat on the size of the gall bladder.

3. The time required to virtually empty the gall bladder of its fluid contents ranges from sixteen minutes to four and one-half hours.

4. The proof that the gall bladder is evacuated by a meal of egg yolk is based not merely upon the disappearance of the shadow, but upon the smallest volume recorded before the

shadow disappeared or before the series was discontinued. In no case was the lowest volume over 4.5 cc.; in nineteen out of twenty-four cases it was less than $2\frac{1}{4}$ cc. and in six cases it was half a cubic centimeter or less.

5. The first phase of contraction is the most important, since it discharges three-fourths of the volume of the gall bladder within thirty-two minutes after a meal (average of last eighteen cases).

6. Other fatty foods, like milk and cream, cause as great a first discharge as egg yolk, but they induce no subsequent phases of contraction; from which it is inferred that the gall bladder is a trigger mechanism which is set off equally well by any of a large number of appropriate foods, but that subsequent phases and complete emptying depend on a food which is retained in the stomach sufficiently long and is of such a nature as to induce repeated phases of contraction by repeated spurts of chyme. In support of this view, it was found that repeated injections of small amounts of egg yolk into the duodenum each induced but a single phase of contraction.

7. A study of the rate of emptying has disclosed marked sex differences, the gall bladder emptying much faster in women than in men.

8. The first phase of contraction is divided into three parts: 1) the 'initial response'—of two minutes' duration, in which more bile is discharged than in any subsequent two minutes; 2) the 'two-minute pause,' characterized by filling of the gall bladder; and, 3) the 'principal period of discharge,' which empties more than half the contents of the gall bladder.

9. On the basis of McMaster and Elman's experiments with dogs, the 'initial response' is interpreted as a contraction of the gall bladder induced (or preceded) by the sudden opening of the sphincter; and the 'two-minute pause' as being due to the resumption of tonus by the sphincter.

10. Since, in dogs, it has been established that the smell of food opens the sphincter, the theory that the 'initial response' to ingestion of food in man was due to the opening of the

sphincter was tested by submitting patients to the odor of fried meats. Four out of eleven cases responded by an average initial discharge of 4.2 cc. bile; four others showed a delayed contraction.

11. Distention of the duodenum with air produced a discharge of $4\frac{2}{3}$ cc. (average of three out of four cases).

12. Merely drinking a glass of water resulted in an initial discharge of 5.57 cc. (average of eleven out of fourteen cases).

13. Each of these three agents (smell, distention with air, and drinking of water) thus reproduced the 'initial response' induced by ingestion of food. But a few individuals in each case also showed a 'two-minute pause' and a short 'principal period of discharge,' neither of which was as pronounced as after yolk.

14. Further evidence of dual regulation of the evacuation of bile is afforded by an hour-glass gall bladder, in which the gall bladder continued to contract while the sphincter remained closed.

15. These observations suggest that the chief problem which awaits solution is an analysis of the regulation of the flow of bile by the mechanism at the outlet of the common bile duct.

APPENDIX

So many requests have been received for information about the graphic method used in computing the volumes of the cholecystograms that it has seemed desirable to give a more detailed description of it. Before doing so, however, it is desirable to speak of two reservations which must be made in regard to its use. In the first place, only the fundus and body of the gall bladder can be computed, since the neck, as a rule, lies in another plane. Secondly, the method assumes that the gall bladder is round in cross-section—an assumption which is warranted when the organ is contracting, but which is subject to error when the organ is relaxed sufficiently to be flattened. As a method of measuring absolute volume, therefore, its chief value consists in measuring the response of the gall bladder to those stimuli which induce contraction. But even in other cases its value is nearly as great, since it records the relative changes in the condition of the organ. Thus, supposing a contracting vesicle suddenly relaxes and flattens, the ascertained volume of the flattened organ, when computed as if it were round in cross-section, is larger than its absolute volume, but by this very exaggeration attention is called to the change from contraction to relaxation. This being understood, the method serves a double purpose—it provides a sensitive means of detecting relative changes in the condition of the organ and records its changes in absolute volume when under contraction. The following description of the method is by Prof. Elliott T. Adams, to whom I am greatly indebted for the application of this engineering method to a biological problem.

Figure 19 shows a sample tracing of a gall-bladder shadow which has been bisected longitudinally (II). Draw a line CD parallel to the long axis AB . Erect a series of perpendiculars to these parallel lines. On perpendicular e lay off a distance above the line CD which is numerically equal to the area of the cross-section of the bladder at the level where its tracing is intersected by this perpendicular. This may be rapidly done with the aid of graph III, which is so constructed that the abscissa of any point is numerically equal to the area of a circle having a diameter equal to the ordinate. Proceed in a similar manner with the other perpendiculars. Pass a smooth curve, CFD , through the points so obtained. It follows from the

elementary calculus that the area enclosed by the line CD and the curve CFD will be numerically equal to the volume of the gall bladder. This area is consequently measured with the aid of a planimeter.

The perpendiculars do not need to be spaced at regular intervals along the axis AB . Theoretically, it is better to space them so that they are closer together where curve CFD is the more sharply curved

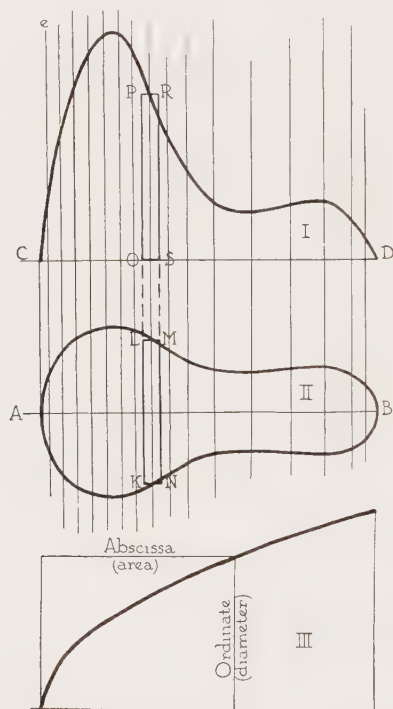


Fig. 19 Chart illustrating graphic method of computing volumes of the gall bladder.

or where it has the steeper slope. But in practice it is nearly always sufficiently accurate to use coordinate paper with ordinates $\frac{1}{5}$ of an inch apart.

The theory of the method is very simple. Consider the shape of the bladder to be approximated by a series of circular discs. Let $KLMN$ represent one of these discs as seen from the edge. Let the height OP of the rectangle $OPRS$ be numerically equal to the area of the base of the disc. Let the width of the rectangle equal the thick-

ness of the disc. Since the volume of the disc will be equal to the area of the base multiplied by its thickness, it is obvious that the area of the rectangle *OPRS* will be numerically equal to the volume of the disc. The volume of the remaining discs may be similarly represented by rectangles. The integrated area of these rectangles will then be approximately equal to the volume of the bladder. Their area will also be approximately equal to the area enclosed by the smooth curve *CFD* and the line *CD*.

It is now possible to consider that the discs are so thin and, in consequence, the shape of the gall bladder so closely approximated that the difference between the integrated volume of the discs and the volume of the gall bladder is less than any assignable quantity. A little thought will serve to show that when the discs are so extremely thin, the integrated area of the rectangles which represent their volume will differ from the area under the smooth curve by less than any assignable quantity. Hence it follows that the area under the smooth curve is numerically equal to the volume of the bladder.

A somewhat similar procedure is followed when the long axis of the cholecystogram is curved, but the more complicated theory of it is omitted from this account in the interests of brevity. When the units of volume are measured in inches, multiplying the results by 16.4 gives the equivalent volume in cubic centimeters. Finally, correction for magnification by the x-ray machine must be made: in our case it was necessary to multiply the volume by 0.9. Thus determined, computation of the volume of the twenty-odd cholecystograms in each series can be completed in from three to five hours, depending upon the size of the shadows. But since it takes several hours to x-ray the patient, and several additional hours to make accurate tracings, a total of ten to fifteen hours' work is required for each series—a circumstance which would seem to preclude its use as a clinical method.

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PLATE I

EXPLANATION OF FIGURES

(Selected cholecystograms of hour-glass gall bladder, illustrating various phases of contraction and filling during two days in which case *D. W. R.* was under observation. For interpretation of shape see additional outlines of same case, figure 9; for location in contraction curve see figure 10.)

20 Visualization of gall bladder just preceding meal of egg yolk and cream (fifteen and two-thirds hours after oral administration of dye). Note heaviest concentration of dye in lower half of gall bladder and gradual dilution in upper half toward neck.

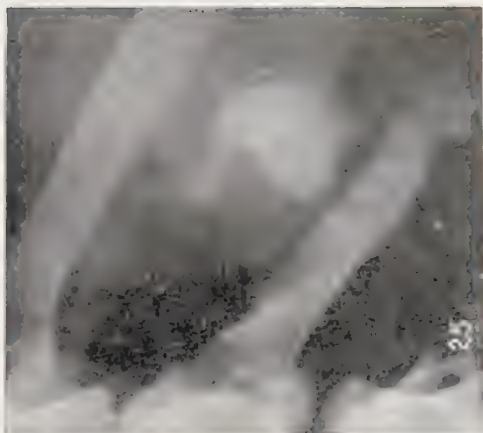
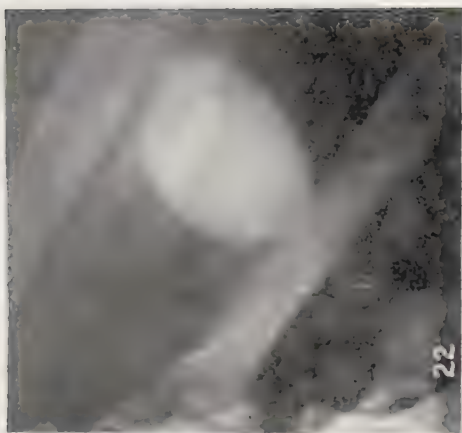
21 Twenty minutes after egg yolk and cream. Note equal concentration of dye in both halves, due to expulsion of bile from lower to upper half, following first phase of contraction against closed sphincter (compare text).

22 Fifty-two minutes after yolk and cream. Gall bladder relaxed and filling; contents of upper half so dilute as to almost prevent visualization; contents of lower half unchanged, owing to length of time required for diffusion of dye through small aperture connecting the two halves.

23 One hundred ten minutes after second meal. Volume of gall bladder still undiminished after two meals (see text).

24 Second day. Four hours after fourth meal. Volume of gall bladder markedly decreased. Cystic duct visualized.

25 Twenty minutes after fifth meal (containing egg yolk). Note decrease in volume after egg yolk on second day as compared with increase in volume during first day.



CYTOLOGY OF THE SYNOVIAL FLUID OF NORMAL JOINTS¹

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ONE HELIOTYPE PLATE (SEVENTEEN FIGURES)

Normal joints and bursae contain a small amount of rather thin glairy fluid which serves to moisten and lubricate the gliding surfaces. This is called synovial fluid, because it resembles the white of an egg in appearance. Relatively little is known of the origin, chemical composition, or cellular contents of the normal synovial fluid. This paper embodies the results obtained by cytological examinations of samples of fluid from a considerable number of normal joints and is an attempt to establish a norm for the synovial fluid of man and the common laboratory animals.

The current opinion seems to be that the synovial fluid contains only the débris cast off from the joint surfaces, and occasional leucocytes or lymphocytes. As far as I can learn, the only investigator who has studied the cytology of the synovial fluid in detail is Hammar(1). In his two classical papers on the finer structure of joints he included a description of the formed elements of the synovial fluid. His method was to wash out the joint with Müller's fluid, which does not cause coagulation of the joint fluid, and to study the sediment from the washings.

In the washings from supposedly normal joints Hammar noted: 1) Large isolated cells which were round or irregular in shape. They contained from one to three nuclei which

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varied in size and structure. The protoplasm was vacuolated and might contain a few fine fat granules. He believed these to be more or less modified desquamated synovial cells. 2) Smaller round cells with a small amount of protoplasm. 3) The nuclei of encapsulated cells undergoing fatty degeneration. 4) Cell rests, free nuclei, small granular masses of protoplasm without nuclei, and free granules staining like chromatin. 5) Thin membranes staining with carmine and showing a fine striation. 6) Threads with a homogeneous or striated structure which had an affinity for haematoxylin and were devoid of cells. 7) Elastic fibers. 8) Larger tissue masses and broken-off villi of various kinds. 9) Fat droplets. 10) Red blood cells.

Hammar's studies were all made upon fixed material and he was not able definitely to identify the cells in the fluid. However, his observations represent practically all that is known of the cells present in the normal synovial fluid. By means of supravital staining I have been able to identify the various cells present in normal fluid(2) and to determine the numbers of each type present in the fluids of normal joints.

MATERIAL AND METHODS

The greater part of the observations recorded in the present paper were made upon fluids obtained from the shoulder joints of normal rabbits. The knee, while a larger joint, contains much less fluid than the shoulder. To obtain a sample for study, the animal is anesthetized and an incision is made in the skin over the shoulder. The joint line is located by palpation and a small glass pipette is thrust through the muscle and into the joint cavity. The shoulder is then flexed and the intra-articular pressure causes the fluid to rise in the tube.

By the above method, from each shoulder of a normal adult rabbit one is usually able to obtain enough fluid to make a cell count, a smear, and four supravital preparations (about 0.2 cc.). It is important that the fluid be withdrawn immediately after the first puncture, as otherwise the fluid will

be contaminated with blood. For this reason, a given joint can be used only once.

The supravital preparations are made after the method recommended by Sabin(3). Saturated alcoholic solutions of the dyes (neutral red and Janus green B) are kept in stock. Diluted solutions are made by adding a definite amount of the saturated alcoholic solution of the dye to a given amount of absolute alcohol. Clean slides are flamed and, when cool, are flooded with the diluted alcoholic solution of the dye and allowed to dry in a vertical position. This leaves a thin film of the dye on the slide. The strength of the solution to be used for flooding the slides must be determined by experiment. For the study of normal synovial fluid, slides prepared by flooding with solutions containing thirty-one drops of the saturated alcoholic solution of neutral red, or fifteen drops of the saturated alcoholic solution of Janus green to 10 cc. of absolute alcohol, were found to be satisfactory. For double staining of the cells, slides prepared from a fresh alcoholic solution of both the neutral red and the Janus green in the above concentrations were used. The double solution of the stains is not permanent and should be freshly prepared. The prepared slides keep indefinitely. For the study of more cellular fluids, stronger solutions of the dyes must be used.

A small drop of the fluid to be studied is placed upon a prepared slide, covered with a cover-glass, and ringed with a mixture of vaselin and paraffin. The preparation is then placed in a warm box kept at 38°C. and studied under a microscope in this box. The dye on the slide is dissolved by the fluid and taken up by the cells. The Janus green stains the mitochondria, and the neutral red stains the specific granules or segregation vacuoles of the cells. If the dyes are not too strong, the cells remain alive and motile for from one to two hours or more and there is no staining of the nuclei or diffuse staining of the cytoplasm. The Janus green is more toxic than the neutral red and usually kills most of the cells after about half an hour or so. If it is too strong, the nuclei are stained and the cells are killed more quickly.

The cover-glass smears were made in the usual manner and fixed in osmic vapor or dried in air and stained with Wright's blood stain. The cell counts were made in an ordinary blood-counting chamber on the undiluted fluid. It is necessary to stain the nuclei of the cells in order to differentiate them from the fat droplets and connective-tissue débris. For this purpose, a thin film of a solution of a nuclear stain (methylene blue) is permitted to dry on the cover-glass of the counting chamber. This is then inverted over the fluid and the nuclei are stained without diluting the fluid. Four large squares were counted and the sum of the cells in the four squares multiplied by 2.5 equals the number of cells per cu.mm. of fluid. The red blood cells present in the fluid were not counted.

Normal joint fluids from dogs, cats, guinea-pigs, and human beings were also studied for comparison.

TYPES OF CELLS PRESENT

It was found that normal joint fluids were sparsely populated by living cells. The great majority of these were phagocytic mononuclears which resembled the wandering phagocytic cells of connective tissues. Adopting the terminology of Sabin, Doan, and Cunningham(4 and 5), they may be divided into monocytes and clasmotocytes. In addition to the above, there are a certain number of mononuclear phagocytes which are indeterminate in character. A few polymorphonuclear leucocytes are nearly always present. In most joint fluids examined there were also found a few synovial lining cells. More rarely, primitive cells or small lymphocytes were seen.

What may be described as the classical form of the monocyte is a feebly motile cell, 12 to 15 μ in diameter, which contains a large kidney-shaped nucleus. The nucleus contains a faint, rather coarse, chromatin network. When stained with neutral red, the monocyte exhibits a characteristic rosette of minute neutral-red granules which is situated in the hof of the nucleus (fig. 1). The granules are arranged

around a clear area or centrosphere and stain a uniform bright salmon-pink color. If stained also with Janus green, the mitochondria appear as minute green granules scattered irregularly through the cytoplasm. They do not invade the area occupied by the rosette, but some may lie in the narrow zone between it and the nucleus. Not infrequently, a cell contains a group of eight or ten mitochondria near one or both poles of the nucleus.

The above is the typical resting monocyte of the blood and connective tissues as described by Sabin, Doan, and Cunningham(5). In a supravital preparation it is often seen to move slowly about. In motion, the cell usually is elongated and a zone of protoplasm precedes the nucleus. In some instances a thin but definite zone of clear exoplasm can be seen to project beyond the anterior border of a moving monocyte. The granules may stream slowly or change position with a series of short, sharp, jerky movements.

The rapid streaming of the granules and the rapid motion characteristic of the polymorphonuclear leucocyte do not occur in the monocyte. When at rest, the monocyte tends to be spherical in shape, but in motion it is often elongated and the rosette is distorted and out of its normal position in the cell.

In the synovial fluid the typical monocyte as described above is rarely seen. In the resting monocyte of the joint fluid the neutral-red granules of the rosette are larger and less numerous than are those of the blood monocyte (fig. 2). Most of these cells also contain a few medium-sized neutral-red granules which lie in the peripheral zone of the cytoplasm. The nucleus and the mitochondria are similar to those of the classical monocyte. The joint monocytes are nearly always spherical or oval in form and exhibit little or no tendency to movement, either of the cell or of the granules.

Occasionally, in normal joint fluid one encounters a young monocyte. This is a rather small round cell, 7 to 10 μ in diameter, with a relatively large nucleus and a small amount of cytoplasm. The nucleus usually has a shallow indentation

in one side and in this hof is a small collection of minute granules which stain in the characteristic manner with neutral red. These granules are grouped in one or more rows around a small clear area or centriole. The small granular mitochondria are scattered irregularly through the cytoplasm and are less numerous than in the mature form (fig. 4).

The majority of the monocytes of normal synovia are in a state of mild stimulation. They are large round or oval cells 15 to 20 μ in diameter. The large nucleus is eccentrically located and may be round, oval, or kidney-shaped. The characteristic rosette may be present, but more often it is represented by a large spherical mass of neutral-red granules which lies alongside of or in the hof of the nucleus. The granules are mostly minute in size, but a few larger ones are scattered through the mass. Around the periphery of the cell is a zone of neutral-red granules which are irregular in size and distribution. These neutral-red granules may be so numerous that they fill the available cytoplasm and tend to obscure the central mass or rosette. Scattered here and there among the peripheral neutral-red granules there may be a variable number of small- to medium-sized clear vacuoles (fig. 6). As will be mentioned later, these clear vacuoles tend to be smaller and less numerous in the stimulated monocyte than in the clasmatocyte. The mitochondria of these stimulated monocytes tend to be smaller, less numerous, and more difficult to stain than are those of the resting monocyte. A rather frequent form is a cell about 15 μ in diameter, with a large kidney-shaped nucleus. The central mass of neutral-red granules is about twice as large as that of the resting type. Around the periphery of the cell is a single row of rather large neutral-red granules.

Occasionally, one of the monocytes contains two nuclei. When this is the case, the nuclei are situated on opposite sides of the cell with their indentations facing one another, and the rosette or central mass of neutral-red granules lies in the space between them. The two nuclei are usually unequal in size and the binucleated monocytes tend to be rather small

in size and to contain relatively little phagocytosed material (fig. 5). These are probably dividing monocytes, but I have not seen division of monocytes occur in the normal joint fluid.

While three types of monocytes have been described as being present in the joint fluid, it is to be pointed out that these are but different phases in the life-history of a single cell. All transition stages between the young and the mature monocytes occur. Likewise, there are all gradations of stimulation among the mature monocytes. As a matter of fact, the cell which I have described as the resting monocyte of the joint is really a monocyte which is mildly stimulated, as may be evidenced by the fact that it contains one or more neutral-red granules in the peripheral zone of the cytoplasm. From this stage all gradations of phagocytosis may be met with up to the large engorged monocyte which is packed with neutral-red granules.

The clasmatocytes are less numerous than the monocytes and are usually of the mature moderately stimulated type. They are large cells 15 to 25 μ in diameter and contain a relatively small oval nucleus which is as a rule eccentrically located. The cell body is usually oval or round, but many of these cells are elongated and irregular in contour (figs. 9 and 10). They may possess one or more long tapering processes which end in a fine hair-like strand of clear protoplasm. In other instances the processes are short and have a thick rounded extremity (figs. 6, 7, and 8).

The cytoplasm is usually loosely filled with granules and vacuoles which vary in size from minute granules to vacuoles 5 μ or more in diameter. Many of these stain supravitaly with neutral red. When thus stained, they vary in color from light pink to deep red; this variation in the shade of the granules and vacuoles is characteristic of the clasmatocyte as contrasted with the uniformity of the rosette in the monocyte. Another distinguishing feature of the clasmatocytes is that they contain a great many more clear vacuoles than do the monocytes. These vacuoles give the impression that the clasmatocyte has a rather loose structure, while the stimu-

lated monocyte often seems to be packed with neutral-red granules and vacuoles. The granules and vacuoles of the clasmatocyte are scattered irregularly through the cytoplasm and there is no tendency to the definite pattern with a central mass and a peripheral zone of granules, such as is characteristic of the monocyte.

The mitochondria of these stimulated clasmatocytes are very minute granules which are distributed irregularly through the cytoplasm. Because of their small size, they are usually not visible in cells stained with the double stain of neutral red and Janus green. In cells stained with Janus green alone, a few minute granules can usually be seen, but they are smaller and less numerous than are the mitochondria of the monocytes in the same preparation. In some of the stimulated clasmatocytes, however, no green granules can be made out. In these, and also in the clasmatocytes which do contain mitochondria, a few of the small clear vacuoles may take the Janus green lightly. These light-green vacuoles may stain even in the presence of the neutral red in the cell. The neutral red seems slightly to inhibit the staining of mitochondria by Janus green, but has little effect upon the staining of these small clear vacuoles. Whether or not these contain material from degenerating mitochondria, I am not prepared to say. They are too large to have arisen from the swelling of one of the minute mitochondria present in clasmatocytes.

Younger clasmatocytes are occasionally seen in normal joint fluid. They are cells 10 to 15 μ in diameter (fig. 8). The cell body tends to be ovoid, but the protoplasmic processes, as described above in the mature forms, may be present. The nucleus, in size and shape, resembles that of the large mature clasmatocyte. It seems to be characteristic of the clasmatocyte that the nucleus remains about the same, regardless of the size or functional state of the cell. In the monocyte the nucleus tends to increase in size as the cell grows larger.

In the joint fluid these younger clasmatoocytes always contain a few neutral-red granules or vacuoles and usually a few clear vacuoles. This is believed to indicate that they are moderately stimulated phagocytic cells. The tendency of the clasmatoocyte to localize the first phagocytosed material in the region of the nucleus was pointed out by Sabin, Doan, and Cunningham(5). This is well illustrated by the young clasmatoocytes of joints, as in practically all of them there is a row of neutral-red granules along one side of the nucleus. Other than this tendency for the first material to be placed next to the nucleus, there is no evidence of any definite pattern in the cytoplasm of the clasmatoocyte, either old or young, and the granules and vacuoles are scattered irregularly through the cytoplasm. In the young clasmatoocytes the mitochondria are more numerous and more regularly stained than are those of the mature forms.

I have not seen a clasmatoocyte exhibit movement or streaming of its cytoplasm in normal joint fluid. On several occasions, however, I have seen a mature clasmatoocyte change its form by extending and retracting one or more protoplasmic processes. The blunt pseudopodium was bordered by a zone of clear exoplasm, and as the process was retracted this might be drawn out into one or more thin strands which were slowly drawn back to the cell body. The same process might be projected and retracted a second or third time, the cell body remaining stationary by the pointer and elongating and rounding up again. The process occupied two to five minutes and at no time was any visible motion detected in the granules of the cell, though they did of course change position very slowly.

In normal joint fluids the clasmatoocytes were almost always mononuclear. In synovial exudates I have upon two occasions seen mature clasmatoocytes divide, but I have seen no evidence that this occurs in the normal joints.

In addition to the definite monocytes and clasmatoocytes, there are a certain number of mononuclear phagocytic cells in normal joint fluid which I find it impossible to classify.

These cells have characteristics of both of the above types and yet are typical of neither. If one were so disposed they might readily be interpreted as transition stages between the monocytes and the clasmatocytes. They are round or oval cells 12 to 15 μ in diameter, with a large nucleus which is usually spherical in form. They contain no visible rosette or mass of neutral red, and the neutral-red granules and vacuoles vary in size and color as in the clasmatocyte. These are intermingled with clear vacuoles and have no definite arrangement in the cytoplasm. Mitochondria are present, but are not characteristic of either type of cell. They tend to vary in number inversely with the neutral-red granules and vacuoles. In my earlier studies I classed these cells as clasmatocytes. Later I regarded them as senile monocytes. Now I am unable to classify them, but regard them as merely atypical forms of one of the above two types. Some of them are probably monocytes in which the cell is engorged with phagocytosed material and the characteristic rosette is obscured. But in others the cytoplasm contains only a moderate amount of material staining with neutral red, and a rosette, if present, should be easily seen (figs. 11 to 13).

As stated above, the mononuclear phagocytes of synovial fluid are practically always mildly stimulated cells and contain phagocytosed material. This material is in clear vacuoles or granules in the cytoplasm and cannot be identified as cell or tissue débris. Only once in the examination of approximately two hundred normal synovial fluids have I seen a clasmatocyte which contained a red blood cell. On no occasion have I seen a phagocytosed nucleated cell in normal joint fluid. The constant presence of fat droplets in joint fluid suggested that the clear vacuoles in these cells were fat droplets. But smears fixed in osmic acid showed very few cells indeed which contained any visible fat. These were probably clasmatocytes and there were only a few minute droplets in any one cell.

For the most part the phagocytic cells occur singly, but occasionally two or more are stuck together. Some of these

clumps of cells may contain twenty or more cells. When massed together, the cells are difficult to identify, but it may be stated that monocytes, clasmatocytes, and indeterminate cells may all be present in one aggregation.

In most of the normal joint fluids studied a few typical polymorphonuclear leucocytes were present. They were similar to those of the blood of the animal from which the fluid had been taken. Usually, they were in a state of activity with rapid streaming of the refractile granules. As the cell moved in one direction or another, the nucleus was preceded by a broad zone of cytoplasm. The specific refractile granules of the polymorphonuclear leucocytes were not stained, but in some of the cells there were one or more small granules or vacuoles which stained with neutral red. The minute mitochondria were as a rule obscured by the specific granules, but in a preparation well-stained with Janus green some of the leucocytes contained a few minute green granules.

Small and intermediate lymphocytes and primitive cells are occasionally seen in normal joint fluid. The lymphocytes are small cells with a relatively large nucleus and a small ring of protoplasm. The mitochondria are minute granules or short rods and tend to be clumped at one side of the nucleus where the zone of cytoplasm is thickened. The primitive cell is similar to the small lymphocyte, except that the mitochondria tend to be scattered irregularly through the cytoplasm (figs. 14 and 15). Either type of cell may contain one or more neutral-red granules. As transition stages occur between the two types and as the total number of such cells encountered is very small, I have classed them together in my counts. It is also to be noted that transition stages between the primitive cells and the small monocytes may be encountered. None of these cells was seen to exhibit motion in the preparations of normal synovial fluid.

Detached synovial lining cells were present in small numbers in most of the fluids studied. They vary in size and shape from small spindle- or comma-shaped cells to rather

large irregular cells with one or more blunt processes (figs. 16 and 17). The nucleus is rather small, round or oval in contour, and has a prominent refractile outline. The protoplasm has the appearance of ground glass and may contain one or more clear refractile granules or vacuoles. The mitochondria are minute granules which are scattered irregularly through the cytoplasm. They are moderate in number and in some of the cells small clumps of mitochondria were present in the vicinity of the nucleus. No neutral-red granules were seen in any of the synovial lining cells, but some of them contained a few small clear vacuoles. None of these cells was seen to move while under observation nor was any movement of the mitochondria or vacuoles detected. Occasionally, small masses or clumps of these synovial lining cells were seen.

As was stated above, red blood cells were present in every synovial fluid studied. They are not sufficiently numerous to tint the fluid, but about equal the number of nucleated cells present. They have the appearance of the normal erythrocytes of the blood and no crenated or laked red cells were encountered. It is believed that these cells in small numbers are constantly present in synovial fluid and that they were not introduced by hemorrhage incident to the joint puncture. The finding of a clasmatocyte which contained a recently phagocytosed red cell is offered as further proof that red cells are normally present. This clasmatocyte was seen a few minutes after the fluid was removed from a normal joint.

Protoplasmic fragments were occasionally seen. It is interesting that these fragments resembled the protoplasm of clasmatocytes and contained clear vacuoles and neutral-red granules and vacuoles of varying color. Although no counts were made, I am of the opinion that these fragments were more numerous in the joints which contained the higher percentages of clasmatocytes. It is probable that most, if not all, of these cell fragments are broken-off processes of the mature clasmatocytes.

Very few naked nuclei were seen. The fragments of connective tissue as described by Hammar(1) were occasionally seen, but were rare. Detached or broken-off villi were not seen in the fluids of the joints studied.

THE NUMBER OF NUCLEATED CELLS PRESENT

The total number of nucleated cells, of all types, present in the fluid from the normal shoulders of a large series of young adult rabbits, varied from 80 per cu.mm. to 375 per cu.mm. In the majority of instances the cell counts ranged from 175 to 225 cells per cu.mm. In the knee joint of a large dog there were 475 cells per cu.mm.

TABLE 1

Differential counts of cells found in normal synovial fluid

	MAXIMUM	MINIMUM	AVERAGE IN FIFTY NORMAL JOINTS
Synovial lining cells	7	0	3
Primitive cells	10	0	1
Polymorphonuclear leucocytes	12	0	5
Indeterminate phagocytic cells	29	3	14
Clasmatocytes	28	4	15
Monocytes	84	42	58

The differential counts were made by the supravital method. For the most part only one hundred cells were counted. The cells are so few that two or even three preparations were sometimes necessary in order to obtain a hundred cells. These counts showed a wide variation in the number of the different types of cell present. In table 1 are given the results of the examination of the fluids from fifty normal shoulder joints of rabbits.

It is seen that the monocytes are the predominating cell in the joint fluid and may run as high as 84 per cent of the nucleated cells. The smallest number of monocytes found in any normal joint was 42 per cent and the average was 58 per cent. The clasmatocytes varied from 4 to 28 per cent

and averaged 15 per cent. The indeterminate phagocytic cells varied from 3 to 29 per cent and averaged 14 per cent. It is thus seen that 87 per cent of the cells in the fifty fluids were wandering phagocytic cells or macrophages.

The leucocytes, primitive cells or lymphocytes, and synovial cells were not constantly present. The greatest numbers found in any normal joint were 12, 18, and 7 per cent, respectively.

It is to be noted that the low cell counts as given above are found only in synovial fluids removed from normal joints of living animals or from normal joints very soon after the death of the animal. After death, cells from the surrounding tissues wander into the joints and the cell content of the synovial fluid is markedly increased. This was shown by making a series of cell counts on the fluid from the excised knee joint of a large dog. The first count made within an hour after the death of the animal showed 325 cells per cu.mm. Three hours after death, the joint contained over 700 cells per cu.mm. and six hours after death, it contained over 2000 cells per cu.mm.

Not only is the total number of cells increased but the differential count is changed. Six hours after death, there were 60 per cent polymorphonuclears and 40 per cent mononuclear phagocytic cells. This indicates that the polymorphonuclear leucocytes to the number of about 1200 per cu.mm. of fluid had entered the joint, while about 500 monocytes and clasmatocytes per cu.mm. had wandered in. Examined by the supravital technique, these cells were living and the leucocytes were motile. There was also a considerable number of synovial-membrane cells (about 10 per cent) in the six-hour fluid and not infrequently two or more of these were clumped, or in a small sheet of detached tissue.

DISCUSSION

It is shown that the synovial fluid of normal joints is constantly inhabited by living cells. In supravital stained preparations these cells can be identified as monocytes, clasmatoocytes, indeterminate mononuclear phagocytes, lymphocytes or primitive cells, leucocytes, and synovial-membrane (lining) cells. Since it has been shown by Cunningham, Sabin, and Doan(6) that the primitive cells develop into monocytes, all clasmatoocytes, monocytes, indeterminate mononuclear phagocytes, and primitive cells may be grouped together as the macrophage series.

The cells of the macrophage series are the most important cellular constituents of normal synovial fluid. In a series of fifty normal synovial fluids 88 per cent of the nucleated cells belonged to this group. The monocytes averaged 58 per cent and the clasmatoocytes averaged 15 per cent. This is approximately the proportion found by Cunningham, Sabin, and Doan in the connective tissues. This is further evidence that joint cavities are clefts in the connective tissue which are incompletely lined by slightly modified connective-tissue cells(7). In addition to the definite monocytes and clasmatoocytes, there were a number of macrophages which were not typical of either type. These cells I have called indeterminate macrophages. I do not regard them as transitions from monocytes to clasmatoocytes, but believe that they are either monocytes or clasmatoocytes which were once typical of the type to which they belonged, but have later reacted to stimulation and have lost their characteristic morphology.

Practically all of the macrophages in the normal synovial fluid are mature cells and are moderately stimulated. As I have shown elsewhere, their function is to remove tissue waste or foreign matter from the joint cavity(8).

Primitive cells and small lymphocytes were both described as being present in normal joints. As yet we have no adequate method of differentiating these two types and our decision as to whether a given cell is a primitive cell or a lymphocyte rests largely on where the cell is found. In the

joints I have consequently classed all of the cells of this type as primitive cells, because I have noted transition stages between these cells and monocytes in the joint fluid. The reticular cell, described by Cunningham, Sabin, and Doan(6) as the precursor of the primitive cell, was not seen in the synovial fluid.

A moderate number of red blood cells are normal constituents of synovial fluid and in most instances there are also a few leucocytes.

It was rather surprising that so few of the detached synovial-membrane cells were present in the joint fluid. The highest number present in any normal joint fluid was 7 per cent of the nucleated cells. Degenerating cells and cartilage cells were practically never seen. These findings suggest that friction and attrition of the joint walls play little part in determining the cell content of normal synovial fluid.

The fluids from the normal joints of the other laboratory animals and of children contained the same types of cells and in about the same number and proportions as did the synovial fluids of the rabbits.

SUMMARY

Normal joint fluid contains from one to three hundred living cells per cu.mm. and also about an equal number of red blood cells, and a small amount of fat and tissue debris. By means of supravital staining it was possible to classify and make differential counts of the cells in normal synovial fluid as follows: monocytes, 42 to 84 per cent; elasmatoocytes, 4 to 28 per cent; indeterminate macrophages, 3 to 29 per cent; primitive cells, 0 to 10 per cent; leucocytes, 0 to 12 per cent; and synovial lining cells, 0 to 7 per cent. The above cells are described and pictured and their characteristics are given. After death, the cell content of the synovial fluid is considerably increased and altered in character by the wandering into the joint of cells from the surrounding tissues.

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PLATE 1

EXPLANATION OF FIGURES

The figures are drawings of living cells, most of which were stained supravitaly with neutral red and Janus green. The salient features of the cells were outlined with a camera lucida and the details were then drawn in as accurately as possible. The magnification was that obtained with a 2-mm. objective and a no. 6 ocular; it is indicated by a drawing of a normal red cell of rabbit's blood in the upper left corner of the plate.

In the drawings clear vacuoles are white; neutral red is indicated by varying shades of dark gray; Janus green (mitochondria) is black. The cytoplasm is shaded more than the nucleus and a nuclear structure is indicated, but is not intended to be an accurate representation of any definite chromatin net. The outlines of the cells and of the nuclei are slightly emphasized.

1 Normal monocyte from the blood of the rabbit.

2 and 3 Usual type of stimulated monocytes found in the normal synovial fluid.

4 Young monocyte.

5 Small monocyte containing two nuclei.

6 Stimulated monocyte containing several clear vacuoles and relatively little neutral red.

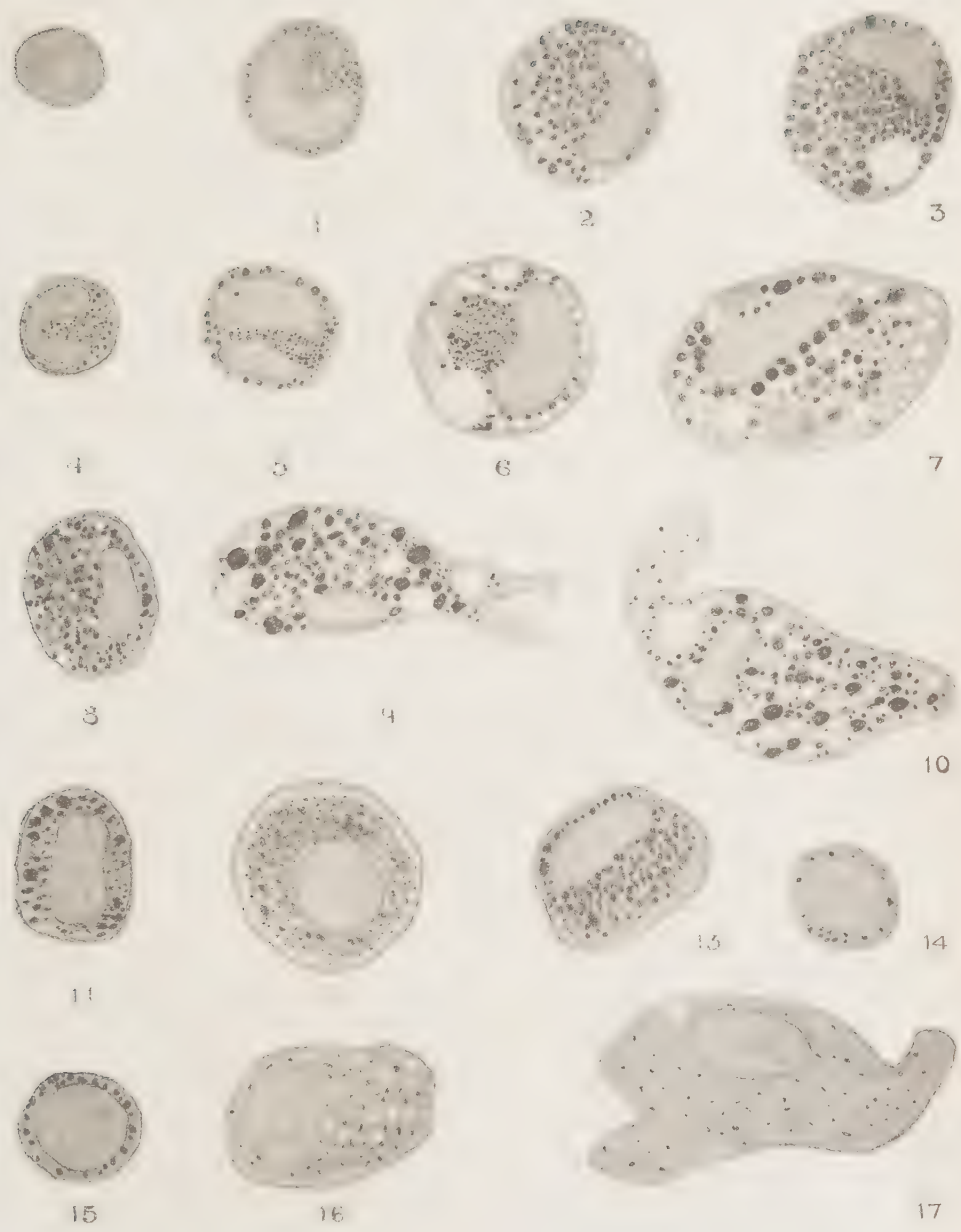
7, 9, and 10 Usual types of elasmatoocytes found in normal joint fluid. The cells shown in figures 9 and 10 are those which may be seen to change their form while under observation.

8 A young elasmatoocyte.

11, 12, and 13 Indeterminate macrophages. Figure 12 is probably a monocyte and the other two are probably elasmatoocytes.

14 and 15 Primitive cells.

16 and 17 Synovial lining cells. The cell shown in figure 16 contains the maximum amount of clear vacuoles found in this type of cell.



God sr
illr

spread
red

A MICROSCOPIC STUDY OF PANCREATIC SECRETION IN THE LIVING ANIMAL

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ONE COLOR PLATE (ELEVEN FIGURES)

It is natural to wish to look into the body and observe directly what is happening under various conditions of health and disease. The most recent advance in this direction is that introduced by Sandison ('24, '28 a, '28 b). It consists of a chamber, built of two layers of celluloid, which is sewed into the rabbit's ear in such a way that the central artery runs in a groove between them. In the course of a few days the capillaries grow out in thin sheets between the celluloid layers and may be studied with oil-immersion objectives under the most favorable optical conditions. It was in the hope that somewhat similar windows could be constructed which would permit the examination of living gland cells that the following experiments were made.

EXPERIMENTS

The pancreas was selected for study because of its ready accessibility and the ease with which its cells may be spread in a thin layer for microscopic observation. In the latter respect it is more convenient than the salivary glands, which, moreover, from the cytological point of view, are less well known.

At first, celluloid chambers were constructed like those of Sandison, but modified so that they could be sewed between the layers of muscle and skin of the anterior abdominal wall. The idea was to place in the chambers some of the small

masses of pancreatic tissue which occur along the greater curvature of the stomach, care being taken to keep their blood supply, nerves, and ducts intact, and then to use lateral illumination for their study. But it was found, in practice, that although the circulation could be maintained, it was difficult to find areas of tissue thin enough for satisfactory study, and the movements of the abdominal wall during respiration proved troublesome.

Other chambers were built, always like those already mentioned, which, when inserted in guinea-pigs and rabbits, under ether anesthesia, would not lie flush with the surface of the body, but projected out from it vertically a distance of 2 or 3 cm. In this way the included parts of the pancreas could be held stationary and studied by direct-transmitted light. But it was difficult over a period of days to maintain a temperature equivalent to that within the abdomen, and the conditions for prolonged study were still far from ideal, so that attempts to implant permanent windows were discontinued, at least for the time being.

Several experiments were then made with large frogs, which, being cold-blooded animals, did not require special apparatus for temperature regulation. It was found that the acinous cells could be readily observed with high magnifications in the thin patches of pancreas lying in the mesentery of the duodenal loop, when these were carefully drawn out from the abdomen and mounted, with circulation still intact, between slides and cover-glasses or sheets of celloidin. Since, however, the same could be accomplished just as easily with white mice, it was decided to base the observations on experiments with the latter animals. The details of the method finally adopted are as follows.

A mouse is anesthetized with ether and fastened to a flat piece of cork. The cork had been so fashioned as to fit onto a brass plate soldered onto the mechanical stage of a microscope. A space had been cut in both cork and brass to permit the raising of the condenser close enough to the preparation to insure a sufficient amount of light, for which a carbon-arc lamp was indispensable.

An incision is made in the left side of the abdomen large enough to allow the spleen and the attached portion of the pancreas to be gently pulled through. The latter is spread on a warmed glass slide, fastened to the cork, and covered with a thin cover-glass. Warmed physiological saline solution is then drawn between the layers of glass, and absorbent cotton, moistened with the same solution, is packed into the wound.

The preparation may be observed in a warm chamber, but it is more convenient to rely upon the heat from the carbon lamp, because it is awkward to control the ether anesthesia in the warm chamber. The objection to urethane as a substitute for ether is that it makes the reaction of the pancreas to pilocarpine more sluggish. But urethane proved useful in a determination of the length of time that the animals would live and during which the cells could be continuously observed. Six mice prepared in this way survived for from eighteen to fifty hours under urethane.

The experiments were planned to determine the response of the living acinous cells of the pancreas to the subcutaneous and intraperitoneal injections of secretin, pilocarpine, and neutral red.

OBSERVATIONS

It will be noted that the technique adopted is in principle the same as that used by Kühne and Lea and by Mathews, fifty-two and twenty-nine years ago, respectively. These observers likewise made studies of the pancreas drawn out from the abdomen and mounted between slides and cover-glasses, with the circulation still intact. The results about to be described represent, therefore, a long-postponed continuation of their admirable researches, with the help of greatly improved optical apparatus.

In such preparations the secreting cells can be very clearly seen with the aid of the best apochromatic oil-immersion lenses and good illumination, both oblique and direct. The mechanical stage permits delicate movements of the field of

vision at will. The general appearance of the cells is illustrated in the accompanying plate. The blood circulates very rapidly in the capillaries, at their base, and the platelets can be seen with ease. Whenever the circulation became sluggish or ceased, the observations were discontinued. The outlines of the nuclei are sharp but faint. The cell membranes are usually visible throughout their whole extent, but are distally somewhat masked by the densely packed zymogen granules within the cells; the mitochondria can only very rarely be seen. Indeed, their refractive index seems to be almost identical with that of the cytoplasm. The curious increase in their visibility reported by Cowdry ('18) in still living cells teased apart and deprived of their circulation has evidently not yet set in. No traces of chromidial substance were detected, although in fixed preparations this substance may be revealed in large quantity in the proximal parts of the cells. Even with the best cardioid condenser and strong light, no light-reflecting surfaces could be made out in the space where it is known to occur. It is, therefore, probable that in the living state the chromidial substance exists in the form of an amorphous fluid deposit, as claimed by Cowdry ('24). Neither could a formed Golgi apparatus nor a system of canals be distinguished in the region between the nucleus and the masses of zymogen granules.

When the cells of a pancreas which has not been placed under the influence of pilocarpine or secretin are examined, it is often necessary to search a considerable area of tissue before any movement of the zymogen granules can be detected, but movement in a proximodistal direction is always seen eventually in a few of the cells. The act of discharge was found to be exhibited by a larger percentage of the cells and to be accelerated as a result of the intraperitoneal or subcutaneous injection of those two excitants.

An attempt was made with the skillful assistance of Mr. Rosenberger to record by moving pictures taken at a magnification of 1000 diameters the changes in the position of the granules and the alterations in shape of the cells dur-

ing the process of secretion. Really instructive films were not secured, probably because the light had to pass in all cases through several cells superposed on each other owing to the characteristic grouping of the cells making up the acini. It was also a difficult problem in itself to record simultaneously the very rapid movements of the corpuscles and the much slower movements of the granules. But the results did indicate that further trials should be made.

In order to depict at least the principal stages in the discharge of secretion, camera-lucida drawings were carefully made of the same cells over definite intervals of time. In this way many drawings were made, from which those representing three continuous observations were selected as illustrative of the process of secretion in the living animal (figs. 1 to 3, 4 to 8, and 9 to 11).

Two acinous cells are represented in figure 1 and the other cells comprising the acinus are outlined for topographical purposes, but are omitted in figures 2 and 3. Seven minutes after the intraperitoneal injection of a small dose of pilocarpine, a few vacuoles appeared in their distal cytoplasm (fig. 2). These vacuoles were larger and less refractive than the zymogen granules, and are represented in a lighter shade of gray. They increased in size and passed out of the cell into the lumen of the acinus. The appearance of the same two cells fourteen minutes after the injection of pilocarpine is illustrated in figure 3. Several zymogen granules have also made their way into the lumen. The secreting cells have not shrunk appreciably.

More complete discharge of vacuoles and zymogen was observed in another experiment (figs. 4 to 8). In this case the mouse received 1.5 cc. of a 1 per cent solution of Gr  bler's neutral red forty minutes before it was prepared for observation. When first examined, the cell had the appearance indicated in figure 4. Pilocarpine was then given intraperitoneally and seven minutes later a marked change was seen (fig. 5). As in the foregoing experiment, this consisted of the development of vacuoles in the area of the cell occupied

by zymogen granules. Four of them appeared in situations near the lateral and distal margins of the cell. They increased greatly in size as before, the growth being gradual and progressive in character, not irregular or interrupted. The condition attained ten minutes after figure 5 was outlined is indicated in figure 6. It will be noted that one of the vacuoles (on the right) has almost escaped. Eight minutes later, or twenty-five minutes after the administration of pilocarpine, the largest vacuole was expelled from the distal extremity of the cell, carrying with it a number of zymogen granules (fig. 7). Reference to figure 7 will show that when this happened the distal margins of the cell retracted, suggesting that a portion of it had been almost pinched off. In figure 8, drawn after a further interval of fifteen minutes, one vacuole is represented on the left in the act of passing into the lumen. It is definitely constricted.

While these events were taking place, the cell was under continual and very close observation. The zymogen granules were never seen to arise *de novo*, and no transitions could be found in this experiment, nor in many others like it, between the brilliantly colored neutral-red granules and vacuoles and the zymogen. The neutral-red-stained granules are smaller than the zymogen granules, but when, as is often the case, they are embedded in red-tinted vacuoles the discrepancy in size is not so marked. Zymogen granules were never seen to be lightly colored by the stain. Yet the neutral-red granules and vacuoles are certainly grouped in the intermediate zone of the cell in which zymogen formation must be actively taking place. Since the theory of Parat and Painlevé ('25) and their associates that the Golgi apparatus results from the action of silver and osmium upon these neutral-red-stained bodies seems to have been confirmed by Covell and Scott ('28) and by Cowdry and Scott ('28), it would appear unlikely that zymogen granules are formed from the material making up the Golgi apparatus—a hypothesis advanced by Nassonov ('23), Bowen ('23), and others.

The growing vacuoles seem to be formed by a kind of liquefaction of the zymogen granules like that which is known to occur soon after they are extruded into the lumen of the acinus.

The membrane of the secreting cells evidently possesses a definite tensile strength, but its actual physical make-up is beyond the scope of microscopic inquiry. Just how the vacuoles and zymogen granules passed through it could not be determined. The question is more difficult to answer for the zymogen granules than for the vacuoles, because of their smaller size. But inspection of the vacuole on the left-hand side in figure 6 suggests that the cell membrane is pushed out ahead of it, and that the membrane forms a close investment along the emerging sides of the vacuole. It is possible that, when the vacuole finally leaves the cell, the membrane, being drawn together at the point of exit, coalesces so that at no time is its physical continuity impaired. The time required for transit is very brief, perhaps shorter for the zymogen granules than for the vacuoles. At one moment, they are inside; at the next, without. This question of the mode of exit of secretion is one of those questions that similar methods for the direct observation of living cells, in the hands of others, may eventually answer. The present communication is, in a sense, a preliminary one.

Finally, I wish to report that other experiments made in the same way have revealed the occasional passage of materials in the opposite direction, that is to say, through the proximal margin or base of the cell toward the blood stream. It is usually necessary to examine several animals before this is clearly seen. The phenomenon is represented in figures 9 to 11. Soon after the observation was commenced, eleven granules were observed in the base of the cell and unquestionably within it, as careful focusing, aided by dark-field illumination, showed (fig. 9). They were seen to change their relative positions slowly from time to time, being limited in their movements by the cell membrane. Gradually, some of them approached the cell membrane and passed through it into

the intercellular cleft between the acinous cell and the endothelium of an adjacent capillary in which the blood cells were actively circulating. The state of affairs after the lapse of thirty minutes is illustrated in figure 10. Two granules seem to be within the intercellular space and one (to the right) appears to be entering it. As soon as they left the cell they moved a greater distance and traveled more freely.

Why granules should leave the cell in this way it is difficult to say. It is conceivable that they are forced out by mechanical pressure of some kind arising from the unusual position in which the pancreas is held while the observations are being made. This possibility is merely mentioned; it is not advocated. On the other hand, the process may be really a normal one, because Horning ('25) has described appearances in fixed preparations which he believes to indicate that granules having the same staining reactions as zymogen are discharged from this pole of the cell. He has even gone further and suggested that their passage might constitute a kind of internal secretion of trypsin. Chemical evidence pointing to the existence of some such mechanism is advanced by Epstein, Rosenthal, Machling, and Beck ('25). The granules in question may, however, be simply of fatty nature, for it is recognized that droplets of fat are prone to occur in the proximal parts of the acinous cells (Mathews, '99, and Saguchi, '20). In my experience the cautious addition of a little osmic acid causes them to blacken, while the zymogen granules become only pale yellow (fig. 11).

CONCLUSIONS

During the process of secretion, the living acinous cells of the pancreas may be seen to discharge both zymogen granules and fluid vacuoles into the lumen of the acinus. The rate is increased by dosage with pilocarpine and secretin. The intracellular vacuoles appear to be formed by a gradual liquefaction of the zymogen granules. No transitional stages could be detected between neutral-red granules and zymogen.

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PLATE 1

EXPLANATION OF FIGURES

1 to 3 Camera-lucida drawings, illustrating stages in the secretory activity of two acinous cells. Figure 1 shows the relation of these cells to others comprising the acinus and their appearance at the time the observations were commenced. Figure 2 was drawn seven minutes after the intraperitoneal injection of pilocarpine. It shows the formation of small vacuoles in the distal parts of the cells. Figure 3 represents the alterations which had taken place after a further interval of seven minutes. $\times 1000$.

4 to 8 Camera-lucida drawings of the secretory process in a cell vitally stained with neutral red. Figure 4 represents the position of the cell observed in relation to two neighboring cells and the number and distribution of neutral-red granules forty minutes after the injection of a 1 per cent solution of neutral red. In figure 5 four small vacuoles are shown. This drawing was made seven minutes after the administration of pilocarpine. Figure 6 shows the increase in size of the vacuoles and their positions ten minutes later. Figure 7 illustrates the stage in secretion twenty-five minutes after pilocarpine was injected. Several vacuoles and granules have been released from the cell. The apex of the cell is altered in shape. Figure 8 represents the condition of the cell fifteen minutes later. The neutral-red granules and nucleus have slightly shifted in position. $\times 1500$.

9 to 11 Camera-lucida drawings of a cell observed over a period of one-half hour and the reaction of the basal granules to 2 per cent osmic acid. Figure 9 illustrates the position of a cell in relation to two other acinous cells and the number and positions of granules between the base of the cell and nucleus. Figure 10 shows the change in position after one-half hour. Figure 11 shows that these blacken with 2 per cent osmic acid. $\times 1000$.

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2

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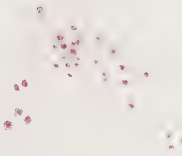
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THE METHODS FOR THE DEMONSTRATION OF
THE GOLGI APPARATUS

VI. PROTOZOA. THE VACUOME. PLANT TISSUES

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METHODS FOR PROTOZOA

A logical review of the methods for demonstrating the Golgi apparatus in various kinds of Protozoa is in the beginning

rendered all but impossible by the simple fact that no basis for the identification of what may or may not be Golgi material in Protozoa has yet been agreed upon. Nor is this disagreement one involving merely two major points of view, but so divergent are opinions that scarcely two workers can be found with anything approaching identity of opinion. This condition arises primarily from the fact that the specialized Golgi-apparatus methods are not specific, and in cases otherwise doubtful the results, for example, of silver or osmium impregnation, are well-nigh useless for purposes of critical identification. Even this difficulty would, however, not be so baffling, if the various structures that may be thus demonstrated were in themselves well enough understood to permit a process of elimination with a subsequent residuum that could be referred to a common Golgi basis. But at the moment the whole problem is in confusion, and it is impossible to give any general rules for demonstrating the Golgi apparatus in Protozoa. I shall, therefore, arrange the data now at hand arbitrarily according to the usual classification of the Protozoa, with a subsequent tabulation of methods and remarks on vital staining.

Any one who may be particularly interested in this problem will find very useful reviews in the recent papers by King ('27) and Hirschler ('27 a).¹ A study of these papers, together with the technical digest in the pages following, should, I think, give pause to any one who contemplates a reconnaissance of the Golgi apparatus in Protozoa without first having served a long apprenticeship in the better-known cells of Metazoa, where at least some general basis of agreement has been attained.

The technical approach to the problem may be made in two ways, according to the nature of the material—by section methods or by cover-slip preparations. Occasionally, material will permit of both manipulations—a circumstance

¹ These papers contain a few references not found in the literature list at the end of this paper, since I have included only papers in which the technical methods of study have been given.

which should be considered as very favorable. The fact that isolated cells often fail to respond to impregnation methods (part I, p. 313) suggests that similar trouble may be expected in protozoan technic, and that, in consequence, treatment of material in bulk with subsequent sectioning might be considered the more desirable. Whether this be true is, however, not yet apparent. In any event, if the material can be treated in bulk, as, for example, some kinds of parasitic forms embedded in the tissues of a host animal, the technic usual in metazoan work is to be followed, including the ultimate handling of the sections.² In the case of cover-slip preparations (smears, etc.), special rules are often unnecessary, but when impregnation, for example, with osmic acid, is to be attempted, some departure from accustomed routine is necessary. These points are dealt with particularly by Hirschler ('27 a). He suggests that in the case of cover-slip preparations the extravagant use of osmic acid for impregnation may be avoided by the following procedure. Clean cover-slips are first dipped in paraffin several times by one corner. Eight or ten of these can now be treated together in a single container, the paraffin preventing adjacent slips from coming into contact with each other. The osmication must be carried out in air-tight containers, as usual. In the case of cover-slips, however, ordinary bottles are very inconvenient, and the most satisfactory receptacle is a small glass weighing bottle with straight sides and a well-ground stopper. Place the battery of cover-slips vertically in the bottle, and if osmication is to be carried on in an incubator, it is unnecessary to immerse more than half of each cover-slip in osmic solution, in order to get an impregnation over the entire slip. The paraffin corner is finally dissolved off in the clearing xylol. Cover-slip preparations have a tendency to flake off, although this is less serious after initial chrome-osmium

²It will be assumed that the reader is already familiar with the general procedure in Golgi-apparatus technic. Special notes of a general character will be found in parts I and III of this series, to which reference may be made by those unacquainted with the required refinements.

fixation than after some other fixing fluids. In general, however, it must be remembered that so little is certainly known as to favorable methods of handling protozoan material that much must be found out by the ingenious worker himself through the tedious procedure of trial and error.

Methods tabulated according to protozoan classes

Sporozoa. I begin with the Sporozoa, because this group was the first in which a Golgi apparatus was described, and more particularly because in this group alone is there anything like agreement as to the correctness of the identification of the material in question with the metazoan Golgi material. This first record is due to Hirschler ('14), who gave a clear-cut account of the Golgi apparatus in *Monocystis ascidia*, together with some excellent figures. His technic consisted in removing small pieces of the intestine of the host (*Ciona*) and treating them according to the Kopsch method (part III, p. 245), as follows:

Fix (and osmicate) in 3 per cent osmic acid at 25°C. for fifteen to seventeen days.

The Golgi apparatus appears in the form of scattered ring or half-ring structures, the blackening of which is not affected by turpentine (part III, p. 265). Their general morphology is likened to the scattered apparatus in the ganglion cells of Crustacea (Poluszyński) and in the male and female germ cells of *Ascaris* (Hirschler). Many other cases of a similar topography of the Golgi apparatus have since been discovered. These bodies cannot be stained by Fe-hematoxylin after Benda fixation, but they appear to correspond in part with the so-called chromidial bodies which can be demonstrated in this gregarine by sublimate-acetic-hematoxylin. By the method of osmication given above, and also by the use of 2 per cent osmic acid in the same way, Hirschler was able also to blacken the mitochondria—in the form of smaller and much more numerous granules, in no way to be confused with the Golgi bodies proper.

In 1924, Hirschler published a brief note on the Golgi apparatus in *Monocystis agilis* (same as in *M. ascidia*) and in *Gregarina polymorpha* and *G. blattarum*. In the latter cases mitochondrial granules could not be demonstrated, but only blackened batonnets (probably Golgi apparatus). The methods used were Kopsch (part III, p. 245), Weigl (part III, p. 249), and Hirschler (part III, p. 261)—no further details being given. In a subsequent paper, however, Hirschler ('27 a) finds that in these latter cases also there are two osmium-reducing components, the mitochondrial type not having been demonstrated in the earlier work.

Hirschler's work on the Golgi apparatus in the Protozoa has been summarized and greatly extended in a recent paper ('27 a). Here he deals with *Monocystis agilis*, *Diplocystis phryganeae*, *Gregarina polymorpha*, and *Clepsidrina blattarum*. In all cases the Golgi apparatus is blackened after proper treatment with osmic acid and appears in the form of scattered vesicular bodies, each divisible into an osmiophilic and an osmiophobic component—as often happens in scattered Golgi bodies in the Metazoa. The chondriome is also demonstrable by similar methods, but only when the osmic content of the initial fixative is much reduced. This paper constitutes one of the most important contributions yet made to the subject, and the methods developed by Hirschler will be found in more detail on page 241. They are essentially modifications of the Kolatchev method (part III, p. 256).

In a series of short contributions culminating in his extended paper of 1926 a, Joyet-Lavergne has dealt at length with the Golgi apparatus (and other cytoplasmic elements) of the Sporozoa, particularly *Coccidia* and *Gregarina*. He finds the Golgi apparatus present in the form of scattered bodies (granules or slightly curved rods). His methods are not given in detail, but he states that the following have been used:

1923—Hirschler's method (part III, p. 270).

1924 b and c—Silver and osmic methods (parts II and III).

1926 a—Kopsch, Weigl, Kolatchev, and Hirschler (part III); and the silver methods of Da Fano and Cajal, which he finds better than that of Golgi (part II).

He notes further ('24 b) that the mitochondria may be stained by Altmann's method after osmic impregnations in which they have not blackened (Hirschler, part III, p. 270). The Golgi bodies in *Aggregata* are found ('24 a) to have the chromophilic and chromophobic portions already noted, the latter showing a clear affinity for acid fuchsin. This material may also be stained by magenta red, after the chromophilic portion has been blackened by silver impregnation ('24 b). In some cases Joyet-Lavergne ('24 c) was able to impregnate the Golgi apparatus in the sporozoan simultaneously with the apparatus in the intestinal epithelium of the host.

Scattered dictyosomes are also reported in a coccidian by King and Gatenby ('23), presumably by silver impregnation.

In *Haplosporidium chitonis* (Neosporidia), King ('26 a) has demonstrated the Golgi material by the Weigl method ('Mann-Kopsch,' part III, p. 249), as follows:

1. Fix for one to twelve hours in the usual corrosive-osmic mixture.
2. Osmicate three weeks in 2 per cent osmic acid.

From this review it may be concluded, I think, correctly, that the Golgi apparatus in the Sporozoa, at least in the Telosporidia, may be regularly demonstrated by the usual methods of silver and osmic impregnation, exceptions being expected in those phases of the life-cycle where heavy peripheral membranes are met with (Hirschler, '27 a).

Sarcodina. The Sarcodina have proved particularly refractory to Golgi-apparatus technic, and thus far little of a positive nature has been learned. Most suggestive, however, is the report of Hirschler ('27 a) on *Endamoeba blattae*. Sections were prepared from material treated as in the case of the Sporozoa (see p. 241 for details). Two types of scattered cytoplasmic bodies are demonstrated by proper osmic impregnation, their morphology being quite similar to the constituents observed by Hirschler ('14) in *Monocystis*. Of

these, the larger, somewhat disc-shaped bodies are identified as Golgi apparatus. This conclusion seems to be a correct one, the morphology of the bodies being exactly what one might expect. Causey ('25), however, finds in *Endamoeba gingivalis* a juxtannuclear, net-like Golgi apparatus, strikingly like that in many metazoan cells. His method was to fix smears in osmic vapor and stain with Regaud's modified Fehematoxylin or Bensley's acid fuchsin-methyl green. It is to be noted that, under ordinary circumstances, these methods would not be expected to demonstrate Golgi material (except in germinal tissues) in metazoan cells—a fact which, coupled with Hirschler's result, leaves Causey's finding in doubt.

Mastigophora. It is in the group of flagellates, where such a variety of cytoplasmic differentiations occurs, that the greatest confusion concerning the Golgi apparatus prevails. It is at present impossible to harmonize the views held by various workers, and it is useless here to attempt more than a summary of observations to date.

Among the first studies of the Golgi apparatus in flagellates was that of Nasonov ('24) on *Chilomonas*. His method was a slight modification of Kolatchev (part III, p. 256), and is given in detail on page 240. He blackened the wall of the contractile vacuole with osmic acid and interpreted this structure as Golgi material in accordance with his more extended results on ciliates (p. 235).

At about the time of Nasonov's publication, Duboseq and Grassé ('24) published the first of a series of papers in which they have upheld the view that in parasitic flagellates the so-called parabasal body or apparatus is actually the homologue of the metazoan Golgi material ('the idiozome of the spermatid' (Duboseq and Grassé, '27)). These workers have found in the parabasal bodies the same suggestive separation into chromophilic and chromophobic constituents. They ('25 b, '27) seem also inclined to include the parabasal bodies (Golgi apparatus) in a larger category related to the chondriome, but this does not affect the primary homology with the Golgi apparatus which they first suggested. The forms

thus far studied include *Janickiella grassii* ('24); *Holomastigotes elongatum* ('25 a); *Pyrsonympha vertens* ('25 c); *Devescovina Hilli* and *Trichonympha Chattoni* ('27).

The methods used by Duboscq and Grassé have not been given in their papers in any detail, and the following notes contain most of the available information. In so far as their interpretation is concerned, the technic already used by earlier workers on the parabasal apparatus may, of course, also be taken into account, but for the present purpose only the technic of Duboscq and Grassé need be considered.

They find ('25 b) that acetic acid must be omitted from the fixative as in the case of Golgi material in metazoan cells. Champy-Fe-hematoxylin is suggested ('25 c), or perhaps best is fixation in osmic vapor followed by Fe-hematoxylin ('27). In general terms, they simply recommend ('25 b) Fe-hematoxylin after osmic fixatives. Of the methods of silver impregnation ('25 b), Cajal gives positive results, but Da Fano is still better. In the case of osmic impregnation, Kopsch or Weigl (Mann-Kopsch) are applicable ('25 b), although the former may give incomplete and apparently damaged results ('25 c).

The question of the parabasal body in parasitic flagellates (*Proteromonas*, *Tetramastix*, *Monocercomonas*, *Trichomonas*, *Eutrichomastix*, *Hexamastix*) is further considered in much detail in a paper by Grassé ('26 a) alone.

He finds that osmic acid, chromic acid, potassium bichromate, sublimate, and, in a less degree, formalin, fix the parabasal apparatus—in other words, the so-called mitochondrial fixatives. Impregnation by osmic acid succeeds with the methods of Weigl (Mann-Kopsch) (see p. 242 for details) and Prenant-Kopsch (part III, p. 247). The trichonymphines are the best material. In the case of *Trichomonas batrachorum* and *Trypanosoma Brucei* successful impregnation occurred only after osmication for sixty days. The methods of silver impregnation (Cajal and Da Fano) have been noted above. Grassé also reports successful use of certain methods for the demonstration of fats. Thus Smith-Dietrich (part V, p. 111) sometimes gives good, positive results, sometimes not, depending on the form. Ciaccio's method is not elective or sharp and was replaced by a modification which stains the parabasal apparatus a bright rose in various flagellates (e.g., *Trichonympha*, *Devescovina*, *Hexamastix*,

etc.), (see p. 242 for technical details). Of general fixatives which may be followed by Fe-hematoxylin, he uses Champy or the following mixture, which may be allowed to act for ten minutes to twenty-four hours:

0.5 per cent Chromic acid,	3 parts
0.5 per cent Osmic acid,	1 part
Acetic acid,	about 5 drops per 100 cc.

He also fixes smears, from which the excess of liquid has been removed, for not more than ten minutes at 40° to 45°C., in:

Trichloroacetic acid,	.5 to 1 gram
Saturated corrosive sublimate,	100 cc.

After washing in running water, mordant for a long time in 1 per cent Fe-alum and stain in 0.5 per cent hematoxylin. This method is particularly good for *Trichomonas*. But the best procedure is to fix, as noted previously, in osmic vapors, and the details of this method are given on page 243. Finally, he uses the method of Drew (see p. 267 for details), with some minor modifications in times and temperatures for which, if desirable, reference may be made to Grassé's original paper.

In two other papers the problem of the flagellate Golgi apparatus is further considered by Grassé ('25 and '26 b), this time in the euglenids. Here there is no obvious parabasal apparatus, and he arrives at the conclusion that its homologue, and that of the Golgi apparatus also, is the stigma or eye spot so familiar in these forms. He finds that this body is blackened, usually imperfectly, by Cajal's silver method, but very sharply by the osmic-acid methods of Kopsch, Weigl, and Kolatchev. The stigma is further very sensitive to acetic acid, and is said to have the duplex structural feature of the Golgi complex in many male germ cells. On the other hand, and contrary to what might be expected from Nasonov's ('24) studies, the contractile vacuole (and reservoir) are never blackened. Grassé's interpretation of the eye spot has been severely criticised by Mangenot ('26), who considers it rather as a modified chromoplast. It is impossible at present to make any profitable comments on this controversy.

Finally, there is the very recent work of Hirschler ('27 a) on *Bodo lacertae*, *Lophomonas blattarum* and *L. striata*, *Trypanoplasma helicis* and *T. dendrocoeli*. The methods used (the same as in *Endamoeba*, noted on p. 230) are modifications of Kolatchev. By varying the percentage of osmic acid in the initial fixative, Hirschler was able to effect a blackening of two different cytoplasmic constituents, and in all cases these are referred to the chondriome on one hand and Golgi apparatus on the other. The basis for this distinction he rests upon a postulated selectivity of osmic acid for the Golgi apparatus after fixation with a fluid rich in osmic acid (for further details see p. 241).

On this basis Hirschler arrives at the conclusion that the so-called ring-body II in *Bodo* is the Golgi apparatus, while the parabasal body is the chondriome (compare Duboseq and Grassé). The ring-body II (Golgi material) is not demonstrated after alcohol-acetic, Carnoy, Schaudinn, or sublimate acetic, but it is stained by Fe-hematoxylin after Flemming, crystal violet after Benda, and Fe-hematoxylin or acid fuchsin after Champy. It is, on the contrary, negative to sudan III after formalin, to Ciaccio's sudan III method, and to Smith-Dietrich (compare reaction of parabasal body, p. 232; see also the table given on pp. 716, 717 of Hirschler's paper). In *Lophomonas* a so-called ring body and characteristic scattered discs are blackened, while the parabasal body is never blackened and scarcely visible in osmicated preparations. The discs, identified as chondriosomes, have a remarkable similarity to the Golgi bodies in *Endamoeba* and would by some workers undoubtedly be so designated. The ring-body which Hirschler identifies as Golgi apparatus is very poorly preserved in osmic-free or in acidified osmic fixatives, but it is well fixed by Altmann, though showing little affinity for the usual stains. In *Trypanoplasma* osmic acid blackens the flagellum sac (blepharoplast) and also scattered disc-like bodies. The latter are very similar to those in *Lophomonas* and again are identified as chondriome, while the flagellum sac is the Golgi apparatus. This structure is also stained by Fe-hematoxylin after Flemming and by crystal violet after Benda.

It will be evident from this work that Hirschler's conclusions are at variance with those of Duboseq and Grassé, and offer no points of obvious comparison with Nasonov's work on *Chilomonas*. One is tempted to suggest that the scattered

bodies which Hirschler has demonstrated in all the forms studied by him (except Bodo) may actually all be Golgi material and so mutually homologous. Such a conclusion would be supported by morphological similarity and would certainly render the comparison of Golgi structures in the Sporozoa, Sarcodina, and Mastigophora much easier. I believe that the possibility of such a common nature should be taken into account in further attempts to disentangle the conflicting views of the Golgi apparatus in Protozoa.

Infusoria-ciliata. There are at present only two views concerning the nature of the Golgi apparatus in the ciliates. One opinion is due to Nassonov ('24, '25), who finds in the wall (or some accompanying structure) of the contractile vacuole, the Golgi material of other types of cells. The other view, held by several workers, considers the Golgi apparatus to be in the form of scattered, discrete bodies. Since some ciliates apparently do not have a contractile vacuole, the two points of view might be to that extent in harmony. But in other instances this cannot be the case.

Nassonov ('24) reported first on *Paramecium caudatum*, *Lionotus folium*, *Nassula lateritia*, *Campanella umbellaria*, *Epistylis gallea*, *Zoothamnium arbuscula*, and *Vorticella* sp.; later ('25), on *Chilodon* sp. and *Dogielella sphaerii*. His methods are a slight modification of Kolatchev, the details being given on page 240. In *Chilodon* the chondriosomes are also blackened, together with the vacuolar rings (Golgi material).

Fauré-Fremiet ('25) reports that the vacuole wall in some of the vorticellids can be preserved in Bouin and then stains clearly, but not very electively, with eosin, indigo-carmin, Mallory, etc. After prolonged osmic fixation or with osmic fixation followed by reduction in pyrogallie acid (part III, p. 244), the wall is tinted in gray. After Flemming or Benda, it does not stain electively either in fuchsin or in crystal violet, but one can use hematein, Fe-hematoxylin, or glychemalum. Fauré-Fremiet does not, however, commit himself to Nassonov's interpretation, but merely corroborates the presence of the vacuolar membrane. A similar result is offered by Young ('24) in the case of *Paramecium*, who worked on sections of material

preserved by Gilson, Bouin, Meves, Benda, Flemming, Altmann, solutions of iodides, etc., followed by Fe-hematoxylin. It may be noted in passing that the methods of fixing and staining used by Fauré-Fremiet and Young would not be expected to demonstrate the Golgi apparatus in the majority of metazoan cells.

Of the forms in which scattered Golgi bodies have been identified, *Opalina* has been most frequently studied, first by Hirschler ('24) and later by King and Gatenby ('26) and Sokolska ('27). Hirschler ('24) first used the Kopsch, Weigl, and Hirschler methods (p. 229), no details being given. He found in *Opalina ranarum* (and *Spirostomum ambiguum*) only one type of blackened body which he was inclined to view as a primitive, composite element combining Golgi apparatus and chondriome. Later, with chrome-osmic fixation and subsequent osmication (probably Hirschler or Kolatchev) Sokolska revealed two types of osmiophilic bodies, one of which was like the Golgi discs of invertebrate Metazoa, the other very small granules, also stainable by Kull or Benda. The latter are apparently the chondriome, the former the Golgi apparatus.

King and Gatenby ('26), also working on *Opalina ranarum*, succeeded in blackening scattered bodies with osmic acid. Their method, essentially that of Kolatchev, is as follows:

1. Fix the whole rectum containing the *Opalina* in Champy for twenty-four hours.
2. Wash in running water for twenty-four hours.
3. Cut the rectum in pieces and osmicate in 2 per cent osmic acid for four days at 30° to 60°C.
4. Wash in distilled water and proceed to section as usual. These bodies may also be demonstrated by Champy-Fe-hematoxylin (King, '26 b).

King ('26 b) also reports scattered Golgi bodies in *Anoplophrya brasili*—a large form parasitic in the gut of *Cirratulus*.

Smear preparations are poor and best results are obtained by fixing the host animals entire, or after cutting into small pieces. Sections are subsequently made as usual. The Golgi elements were never well demonstrated by the methods of Weigl, Kolatchev, or Da Fano, but can be stained clearly with Fe-hematoxylin after Champy fixation. Mitochondrial granules, distinct from the Golgi bodies, can be demonstrated in Da Fano and Champy.

Finally, Lowther ('25) finds in *Uroleptus mobilis* scattered Golgi bodies which may be blackened by the method used by Nassonov, or by Bowen's method (part III, p. 254), or Kopsch (2 per cent osmic for one to three weeks). These Golgi bodies have forms much like those already described for the scattered Golgi elements of other protozoans. Lowther also impregnated the membrane of the contractile vacuole, but rejects the interpretation offered by Nassonov.

*Methods for fresh preparations or intra-vitam study*³

In view of the confusion attending the identification of Golgi structures in Protozoa by the accepted methods of impregnation, etc., it is not to be expected that anything satisfactory can be said concerning the action of vital dyes on the Golgi apparatus. Indeed, the following review offers the same paradoxes of staining behavior *intra vitam* as are found by methods for permanent mounts. With respect to methods for applying vital dyes, there is little to be said beyond the suggestions already given in part I, page 307. A few additional notes of special character as given by individual authors are added in the following résumé.

Sporozoa. In gregarines (*Gregarina cuneata*, *G. polymorpha*, and *Steinina ovalis*) Joyet-Lavergne ('26 b) finds that after the slow action of a sufficiently dilute neutral-red solution,⁴ small red arcs, strongly colored, appear in the cytoplasm, each bordering an ovoid mass weakly colored by neutral red. These bodies correspond exactly with the morphology, size, and location of the Golgi bodies demonstrated by methods of fixation and staining (p. 229), and are similarly interpreted. This is one, perhaps the only, fairly clear-cut case of vital staining of the Golgi apparatus in Protozoa. Nevertheless, neutral red probably does not stain the Golgi material in Metazoa (part I, p. 311; also section on the vacuome, p. 247).

³ See also discussion of the vacuome in Protozoa, page 261.

⁴ See also Mühl on neutral red applied to gregarines.

Sarcodina. Howland ('24) finds that the wall of the contractile vacuole of *Amoeba* takes up neutral red which has been directly injected into the endoplasm. Whether this has any possible relation to Nassonov's conception of the vacuolar wall is very doubtful. Howland herself was not, however, interested in the matter of the Golgi apparatus and does not mention the problem.

Mastigophora. Most of the work done on the Golgi apparatus in Protozoa with vital dyes has been on this group, because many of the structures interpreted by some as Golgi material will respond positively to vital staining or to staining of fresh preparations by toxic dyes.

Grassé ('26 a), working with parasitic flagellates and considering the parabasal body to be Golgi apparatus, finds as follows:

Proteromonas—no result with neutral red, brilliant cresyl blue, and methylene blue; with Janus green, doubtful.

Tetramastix—Janus green 1:30,000 in 0.8 per cent NaCl solution, positive stain.

Herpetomonas—as for *Tetramastix*.

In all these cases the staining of the parabasal apparatus is accomplished only with the greatest difficulty and after several hours' contact with the dye. Best results are obtained by exposing to the air, in a moist chamber, the drop of liquid containing the flagellates (and the stain).

Some other results on the parabasal body, not, however, correlated by the worker with the present problem, are as follows:

Lophomonas (Janicki, '10)—dilute picric acid on living material; but Grassé ('26 a) always got doubtful results by this method.

Herpetomonas (Becker, '23)—parabasal body can be seen with difficulty in the living organism as a more or less refractile spot. It stains after Janus green 1:20,000 in normal saline.

Trypanosoma (Shipley, '16)—cover-slips are coated with a thin, even film of Janus green (95 per cent alcohol, 20 cc.; Janus green, 0.02 gram) so that the color of the dye is almost invisible. Dry and invert over a drop of fresh blood. Or mix blood with equal parts of 1:10,000 Janus green in normal saline. Staining is complete in two to five minutes. The dye takes with remarkable avidity.

Trypanosoma (Werbitzki, '10)—with pyronine, acridine yellow, and oxazine.

Finally, Hirschler's ('27 a) results with vital dyes should be considered in connection with his results after osmic impregnation (p. 234). He made use of neutral red, gentian violet, and Janus green in concentrations of 1:4000 or 1:8000 in 0.65 per cent NaCl solution, with the following results:

Bodo—ring-body II (Golgi apparatus), red in neutral red, negative to dahlia, gentian violet, and Janus green.

Lophomonas—no result ever obtained on the ring-body (Golgi apparatus), though the scattered discs (chondriome) stain intensely with neutral red (p. 234).

Trypanoplasma—flagellum sac (Golgi apparatus) gives negative results with neutral red, gentian violet, and dahlia.

Ciliata. King ('26 b) makes the only definite report.

She mounts Anoplophrya (p. 236) in normal salt or sea-water, where the Golgi bodies can sometimes be seen without staining. Then run neutral red under the cover-slip. The Golgi bodies are stained only after the animal has died. No result was obtained with Janus green. The Golgi bodies in Opalina may also be stained in neutral red (King, '26 b).

In vorticellids (p. 235) Fauré-Fremiet ('25) notes that the wall of the contractile vacuole does not stain with neutral red, brilliant cresyl blue, or Nile blue. But Howland ('24) finds the walls of the canals and contractile vacuoles of Paramecium stained by alizarin blue added directly to a small mass culture. (Compare these results with Nassonov's views, p. 235.)

Special methods for Protozoa

In the preceding review reference has been made to methods chiefly by name. In most cases the procedure has been merely an application of some well-known method to protozoan material. These methods have been discussed in detail in appropriate parts of this series of papers, and references to these parts have been given in the two preceding sections. There are, however, a few modifications which have been developed primarily for work on the Protozoa, and these require special attention.

*Osmic modifications.*⁵ The methods of Kopsch, Sjövall, Weigl, Kolatchev, and Hirschler (part III) have been applied directly to protozoan material by several workers, but experience has shown that, in general, some modification of the initial fixing fluid is desirable in order to achieve a subsequent successful osmication. This was first noted by Nasonov ('24), who observed the same vagaries of osmic impregnation in Protozoa which are so common in Metazoa. He offers two variations of the Kolatchev method, as follows:

Nassonov's modifications (Nassonov, '24):

1. Fix for twenty-four hours in either of the following formulae (based on Champy):

I.	3 per cent Potassium bichromate,	2 parts
	1 per cent Chromic acid,	2 parts
	2 per cent Osmic acid,	1 part
or II.	6 per cent Potassium bichromate,	1 part
	1 per cent Chromic acid,	1 part
	2 per cent Osmic acid,	1 part

In the case of colonies, they may be transferred directly into the fixative. In the case of free-living organisms, they should be squirted from a pipette into the fixing fluid with a quick pressure on the rubber bulb, and care should be exercised that the addition of the culture liquid does not dilute the fixative more than one-third of its volume.

2. Wash with great care in repeated changes of distilled water.

3. Osmicate in 2 per cent osmic acid for three to four days at 35°C. To determine the proper time for osmication, remove samples occasionally and examine under the microscope in toto. If too strongly impregnated, extract in turpentine (part III, p. 265).

4. Wash finally in water and run up through the alcohols. If the objects are not too large or are sufficiently transparent, prepare them as total mounts; otherwise embed in paraffin and section as usual.

Result. By these methods the Golgi material (contractile-vacuole structures) is intensely blackened on a yellowish background. In some cases the granular chondriome may also be blackened (e.g., Chilodon (Nassonov, '25)). Nassonov ('15) recommends Chilodon as one of the best objects for trial, because of the ease with which the reaction occurs in this animal.

⁵All of the precautions necessary for the usual osmic methods must be scrupulously respected (part III, p. 234).

Hirschler ('27 a), after using the methods of Sjövall, Weigl, and Cajal, has finally arrived at the conclusion that the chrome-osmium fixatives (Kolatchev or Hirschler) are preferable. Sjövall and Cajal yield poorer results technically, while after Weigl precipitates may occur. In any case, silver or osmium impregnation may demonstrate not only Golgi apparatus, but also the chondriome. This is, of course, a well-known attribute of both silver and osmium (part II, p. 125, and part III, p. 275), which in the hands of experienced workers rarely leads to confusion. But, in the Protozoa, conditions are quite different, due to the lack of any generally accepted criteria of what is or is not Golgi material. Hirschler proposes to cut this Gordian knot by the use of osmic modifications (variations of Kolatchev or Hirschler, part III) which are presumed in one case to determine a blackening of Golgi material only, in the other a blackening of the chondriome (in some cases with the Golgi material in addition). The determining factor is taken to be the percentage of osmic acid in the initial fixing fluid. If osmic acid is present in the proportion of 1:2, the Golgi apparatus is blackened, if in the proportion of 1:3, the chondriome (and sometimes Golgi apparatus also) is blackened. In case formalin is used as an initial fixative, its concentration follows the same rules, as regards the results of subsequent impregnation (Sjövall method). The validity of this whole proposition has not yet been tested or commented upon by other workers. While of the greatest interest, it seems essential to examine its basis much more thoroughly, especially in view of the morphologically conflicting conclusions to which it leads—as already noted in the preceding sections.

Hirschler's modifications (Hirschler, '27 a):

1. Fix for twenty-four hours in one of the two following formulae:

- | | | |
|--------|---------------------------------|----------------|
| I. | 2 per cent Osmic acid, | 1 part |
| | 1 per cent Chromic acid | } equal parts, |
| | 3 per cent Potassium bichromate | |
| | | 2 parts |
| or II. | 2 per cent Osmic acid, | 1 part |
| | 1 per cent Chromic acid, | } equal parts, |
| | 3 per cent Potassium bichromate | |
| | | 3 parts |

The first formula (1:2) would be for Golgi apparatus primarily; the second (1:3) for the chondriome.

2. Wash thoroughly in running water.

3. Osmicate (probably in 2 per cent osmic acid) for fourteen to sixteen days at 25°C.; or for four or five days at 38° to 40°C.

4. Wash in water as usual, and proceed according to the nature of the object.

Result. As in Nassonov's modifications.

Grassé's modification of Weigl's method (Grassé, '26). Grassé proceeds as follows with parasitic flagellates:

1. Fix for three hours in:

Saturated mercuric chloride,	1 part
2 per cent Osmic acid,	1 part

2. Wash rapidly in distilled water and then osmicate in 2 per cent osmic acid at 30°C. for periods usually over a month. Change the fluid from time to time.

3. Wash and proceed as usual.

Miscellaneous. On the assumption that the Golgi apparatus in parasitic flagellates is the parabasal body, Grassé ('26 a) gives two special methods which may be noted in conclusion. One is a variation of Ciaccio's well-known method for fats; the other, a general staining method.

Grassé's modification of Ciaccio (Grassé, '26 a).

1. Fix in osmic vapors for a variable time, not to exceed twenty seconds.

2. Wash in spring water, ten minutes.

3. Chromatize in 3 per cent potassium bichromate for one hour at 37°C.

4. Rinse in distilled water.

5. Stain in sudan III (saturated alcoholic solution) for a variable time (generally more than twenty-four hours) at 37°C.

6. Mount in Apáthy's gum-and-syrup medium.

Result. Parabasal body is red, if stain is successful (refer to p. 232).

Grassé's general stain for the parabasal apparatus (Grassé, '26 a). Grassé recommends the following method as giving the best and most interesting results on the parabasal body (Golgi apparatus):

1. A small drop of rectal contents, diluted or not with physiological salt, is placed over a 1 per cent solution of osmic acid in a crystallizing dish. The quality of the fixation depends strictly on the duration of this exposure to the vapors. From six to twenty seconds is the requisite time—according to the genus.

2. Then spread the drop out rapidly, and if one fears the floating off of the flagellates, a trace of gelatin water or Mayer's albumen may be added (practically necessary in the case of free-living flagellates). Agitate the slide briskly and evaporate the excess of liquid, the right degree of desiccation being quickly learned by experience.

3. Wash in running water for ten to thirty minutes, depending on the length of fixation.

4. Mordant in 3 per cent Fe-alum for ten to fifteen minutes.

5. Rinse rapidly in distilled water and stain in 1 per cent hematoxylin (made by dilution of a 10 per cent solution of hematoxylin in absolute alcohol), for ten to fifteen minutes.

6. Differentiate under the microscope in 1 per cent Fe-alum for ten to fifteen minutes.

7. Wash a long time in running water, and mount finally in balsam; or, if one wishes to avoid passage through fat solvents, mount in Apáthy's gum-and-syrup medium.

Result. This method demonstrates the parabasal body, mitochondria, and lipoidal constituents of the cell. The results on the Golgi apparatus of sexual cells are similar to the above.

Grassé finds that if the fixation time is incorrect (too long), stable compounds are produced by oxidation which entirely prevent the making of a good preparation. If the final results lack transparency, wash in running water, mordant for four to twenty-four hours in 0.5 to 1 per cent Fe-alum, and after copious rinsing stain in 0.1 to 1 per cent hematoxylin for ten minutes to one hour. Differentiate in 0.5 per cent Fe-alum. For much further information about, and discussion of, Grassé's methods, reference must be made to his original paper.

Check-list of methods

The preceding material has been arranged particularly with respect to the classes and genera of Protozoa and a few special methods. To facilitate more rapid reference to the conflicting data, a tabular arrangement of the more important results according to the method employed is here given. The location of the description of each method as given in these papers is indicated by the Roman numeral (I to VI) of the

part in question, and an Arabic numeral giving the page reference in that part. After each reference to the use of some method on particular protozoans, an Arabic numeral gives the cross-reference page number in this particular paper.

Intra-vitam stains and fresh preparations (I, 306, and I, 312):

- Neutral red*—Mastigophora (Zoomastigoda) Hirschler ('27 a), p. 239: Sarcodina (Rhizopoda) Howland ('24), p. 238: Infusoria-Ciliata (Holotrichida) King ('26), p. 239: Sporozoa (Telosporidia) Joyet-Lavergne ('26), p. 237.
- Janus green*—Mastigophora (Zoomastigoda) Grassé ('26), p. 238; Shipley ('16), p. 238; Becker ('23), p. 238.
- Alizarin blue*—Infusoria-Ciliata (Holotrichida) Howland ('24), p. 239.
- Dilute picric acid*—Mastigophora (Zoomastigoda) Janicki ('10), p. 238; Grassé ('26), p. 238.

Methods of silver impregnation (II, 86):

- General*—Sporozoa (Telosporidia) Joyet-Lavergne ('24 b and c), p. 229; (?) King and Gatenby ('23), p. 230.
- Golgi method* (II, 103)—Sporozoa (Telosporidia) Joyet-Lavergne ('26 a), p. 230.
- Cajal method* (II, 97)—Mastigophora (Zoomastigoda) Dubosecq and Grassé ('25 b), p. 232; Grassé ('26), p. 232; and (Phytomastigoda) Grassé ('25), p. 233: Sporozoa (Telosporidia) Joyet-Lavergne ('26 a), p. 230.
- Da Fano method* (II, 111)—Mastigophora (Zoomastigoda) Dubosecq and Grassé ('25 b), p. 232; Grassé ('26), p. 232: Sporozoa (Telosporidia) Joyet-Lavergne ('26 a), p. 230.

Methods of osmic impregnation (III, 243):

- General*—Sporozoa (Telosporidia) Joyet-Lavergne ('24 b and c), p. 229.
- Kopsch method* (III, 245)—Mastigophora (Zoomastigoda) Dubosecq and Grassé ('25 b and c), p. 232; and (Phytomastigoda) Grassé ('25), p. 233: Infusoria-Ciliata (Holotrichida) Hirschler ('24), p. 236; and (Hypotrichida) Lowther ('25), p. 237: Sporozoa (Telosporidia) Hirschler ('14), p. 228, and ('24), p. 229; Joyet-Lavergne ('26 a), p. 230.

Sjövall method (III, 247)—Hirschler ('27 a).

Weigl (Mann-Kopsch) method (III, 249)—Mastigophora (Zoomastigoda) Duboseq and Grassé ('25 b), p. 232; Grassé ('26), p. 232; and (Phytomastigoda) Grassé ('25), p. 233: Infusoria-Ciliata (Holotrichida) Hirschler ('24), p. 236: Sporozoa (Telosporidia) Hirschler ('24), p. 229; Joyet-Lavergne ('26 a), p. 230; and (Neosporidia) King ('26), p. 230.

Bowen's modification of Weigl (III, 254)—Infusoria-Ciliata (Hypotrichida) Lowther ('25), p. 237.

Hirschler (III, 261) and *Kolatchev* (III, 256) *methods*—Mastigophora (Phytomastigoda) Grassé ('25), p. 233: Infusoria-Ciliata (Holotrichida) Hirschler ('24), p. 236; King and Gatenby ('26), p. 236: Sporozoa (Telosporidia) Hirschler ('24), p. 229; Joyet-Lavergne ('26 a), p. 230.

Prenant-Kopsch method (III, 247)—Mastigophora (Zoomastigoda) Grassé ('26), p. 232.

Hirschler's special modifications for Protozoa (VI, 241)—Mastigophora (Zoomastigoda) Hirschler ('27 a), p. 234: Sarcodina (Rhizopoda) Hirschler ('27 a), p. 230: Sporozoa (Telosporidia) Hirschler ('27 a), p. 229.

Nassonov's special modifications for Protozoa (VI, 240)—Mastigophora (Zoomastigoda) Nassonov ('24), p. 231: Infusoria-Ciliata (Holotrichida and Peritrichida) Nassonov ('24 and '25), p. 235; and (Hypotrichida) Lowther ('25), p. 237.

Chrome-osmic-stain methods (IV, 367):

Flemming-Fe-hematoxylin (IV, 373)—Mastigophora (Zoomastigoda) Hirschler ('27 a), p. 234: Infusoria-Ciliata (Peritrichida) Fauré-Fremiet ('26), p. 235; and (Holotrichida) Young ('24), p. 235.

Benda-crystal violet (IV, 375 and 373)—Practically the same as for Flemming.

Champy-Fe-hematoxylin (IV, 376)—Mastigophora (Zoomastigoda) Duboseq and Grassé ('25 c), p. 232; Hirschler ('27 a), p. 234: Infusoria-Ciliata (Holotrichida) King ('26 a and b), p. 236.

Chrome-osmic-acetic (VI, 233)—Mastigophora (Zoomastigoda) Grassé ('26), p. 233.

Osmic vapor with Fe-hematoxylin, etc. (III, 263, VI, 243)—Mastigophora (Zoomastigoda) Duboseq and Grassé ('27), p. 232; Grassé ('26), pp. 233 and 243: Sarcodina (Rhizopoda) Causey ('25), p. 231.

Methods for fatty materials (V, 108):

Ciaccio or modifications—Mastigophora (Zoomastigoda) Grassé ('26 a), p. 232; Hirschler ('27 a), p. 234.

Smith-Dietrich—Mastigophora (Zoomastigoda) Grassé ('26 a), p. 232; Hirschler ('27), p. 234.

Miscellaneous methods (IV, 377):

Mastigophora (Zoomastigoda) Grassé ('26), pp. 232 and 233; note particularly the use of Drew's method (p. 233).

Bouin and Gilson, with many stains—Infusoria-Ciliata (Holo-trichida) Young ('24), p. 235; and (Peritrichida) Fauré-Fremiet ('25), p. 235.

Critique of the methods for Protozoa

In the midst of such a clash of opinion as to what constitutes the Golgi material, and even more the astonishing variety of methods which have been applied with assumed success, it is indeed rash even to attempt any synthetic suggestions. Nevertheless, there are two features which emerge more and more clearly as one reflects on the accumulated data. One of these is the very general occurrence of an osmiophilic material in the form of scattered bodies which in morphology and especially in behavior to osmic acid have such striking resemblances among themselves and even more remarkable affinities with the well-known Golgi bodies of invertebrate Metazoa. The other feature is the fact that these bodies behave toward osmic impregnation as does the Golgi material in other animal cells, and, what is much more important, that they are not usually demonstrated by any of the customary staining methods. In this, again, these bodies exhibit an arresting similarity to Golgi material in metazoan cells (except germ cells). For these reasons I should be inclined to suggest that in working on Protozoa the presence of such Golgi bodies should be particularly searched for. Experience already shows that failure to demonstrate such bodies would not necessarily prove anything, for the failures of osmic impregnation are notorious and, in the case of Protozoa, the difficulties have turned out to be unusually

tantalizing. On the other hand, the fact that some structure blackens with osmic acid does not in turn necessarily prove anything, for the impregnating capacities of osmic acid are now very much better realized than formerly, due to recent studies on plant cells (refer to p. 268). It may be pointed out finally that a scattered condition of the Golgi apparatus would coincide with much that is known concerning invertebrate cells generally and would be in conformity with the conditions in the Sporozoa, where the identification of the Golgi material seems beyond dispute.

With respect to the methods themselves, a study of available results points most decisively to one conclusion—the method to be used in preference to all others is that of osmic impregnation. This technic has been responsible for practically all of the fruitful work on this problem, and its mastery is certainly a *sine qua non* of successful investigation of the Golgi apparatus in Protozoa. Of the various osmic methods, those of Hirschler and Kolatchev (part III) are unmistakably the most promising, more particularly the variations of these methods developed especially for the investigation of the Protozoa by Hirschler (p. 241) and Nassonov (p. 240). Structures which can be seen by other methods, but cannot be sharply blackened by osmic-impregnation methods, are certainly to be identified as Golgi apparatus with the greatest reserve. Further skillful and tireless application of osmic methods to the Protozoa must, it seems to me, shortly clarify this now perplexing subject. Their use can alone be recommended for general studies, at least on the basis of present knowledge.

THE VACUOME

The term *vacuome* was applied by P. A. Dangeard to the ensemble of vacuoles in a single plant cell, and the study of the exact morphology of the vacuole system was really initiated by this worker in 1916. Subsequently, the problem was taken up by P. Dangeard and, more recently, by Guilliermond, with the result that our knowledge of the vacuoles in plant cells has been much extended and for the first time placed

on a firm morphological basis. These studies have been pursued especially with the aid of vital dyes on living material, but also by making use of the natural coloration effected by anthocyanin and more recently by permanent staining methods especially involving impregnation with silver or osmium. The vital dye most commonly used is neutral red, which has a very elective affinity for the vacuolar contents and has likewise only a slightly toxic effect on the cells themselves. The vacuoles in animal cells were first definitely examined with neutral red and from a point of view similar to that developed by the botanists, by Accoyer in 1924.⁶ This aspect of the problem was quickly followed up by Parat and a number of his colleagues, and, beginning in 1924, a long series of papers has come from Parat's laboratory dealing with the vacuome in animal cells.

From these investigations there has gradually emerged what I may call the vacuome theory. This theory postulates the presence of vacuoles in all plant and animal cells, the contents of which are specifically stained *intra vitam* by neutral red.⁷ This characteristic is to be taken, according to Parat, as the distinguishing feature of the vacuome. While the theory as developed by the botanists has some features which fail to harmonize with the zoological formulation, these differences have been largely ignored by the supporters of the theory as a whole, and, as a matter of fact, are perhaps not of much significance if the general idea at the bottom of the theory be correct.

As to the universal presence of vacuoles in all plant cells there will be pretty general agreement, and a similar thesis may very probably be upheld in the case of animal cells, although the evidence is not by any means so complete. That these vacuoles are very generally stainable by neutral red when the cells containing them are living may also be ac-

⁶Neutral red had been used on animal cells long before this for *intra-vitam* work on what we now know was probably the vacuome—for example, Prowazek ('01).

⁷For data bearing on the stainability of the classical Golgi substance by neutral red, refer to part I, pages 309 and 311.

cepted as substantially correct. That all such vacuoles are expressions of an essentially single category of cytoplasmic structures may likewise be correct, though the evidence of such similarity is rather scanty as yet. As thus stated, the vacuome theory has much of undoubted interest and importance for cellular biology. But its more recent sponsors, particularly Guilliermond and Parat, have gone a step further and made the assertion that the vacuome is actually the Golgi apparatus. In other words, the Golgi apparatus is a system of watery vacuoles quite without lipoidal affinities, and in animal cells never of net-like construction. The figures obtained by silver and osmium impregnation are largely artifacts, due to the deposition of the impregnating medium between, in, or on the surface of the vacuoles proper. In short, the Golgi material of other workers is a myth and most of the methods described in the preceding papers of this series are absolutely unreliable and the truth is not in them. This is, to say the least, a rather large claim, and the whole matter is just now the subject of an acute and pretty general controversy among cellular biologists. Personally, I am entirely unconvinced of the relations between Golgi apparatus and vacuoles as postulated by Guilliermond and Parat. This is not, however, the place to review the arguments pro and con, and reference may be made to my recent review (Bowen, '27 a) for the essential points at issue. To me it seems much more likely that the vacuoles are morphologically distinct from the Golgi apparatus, although in many cases the two may be very closely associated in a topographical way. However, in view of the interest which attaches to the whole matter and particularly in view of the fact that a number of workers consider the vacuome to be the undoubted Golgi apparatus, the methods for its demonstration must here be included.

Considering the extensive literature now accumulated on the vacuome, it is remarkable that the technic for its vital demonstration has never been described except in the most cursory manner. The method partakes of the general in-

definiteness which usually applies to the use of vital dyes as noted in part I, page 307. Much depends on experience and the development of one's personal judgment, and the following details must be applied with these facts in mind. The dye most generally useful in plant cells, and almost exclusively used in animal cells, is neutral red, and this will be given first consideration. For some of the details in technical manipulation I am indebted to a personal communication of Dr. Hope Hibbard, who has for several years been in touch with the methods used in Parat's laboratory.

There are, in the first place, many neutral reds, made by different manufacturers, but the only one recommended (Parat and Painlevé, '25 c) is that made by R. N. de Krall under the general trade name of 'Microcolor.' This is superior to all others and should alone be used for the study of the vacuome.⁸ Make up a stock solution of the dye as a saturated (about 1 per cent) solution in distilled water, normal saline, Ringer, or sea-water, as may be required by circumstances or experience. The solution is made up cold, and the stock bottle is then placed in an incubator at 38° to 40°C., where it may remain indefinitely. The heat is necessary to prevent recrystallization of the dye, which will usually occur in a few days at room temperature. If the solution should recrystallize, put it back in the incubator, and no damage is done. Incubation is not, however, necessary in case distilled water is the solvent. The tissue, plant or animal, is now to be exposed in one way or another to a more or less dilute sample of the stock solution, and after sufficiently prolonged treatment the desired part is teased out on a slide and examined directly. Make all observations in a totally (not merely a partly) darkened room, shading the eyes completely from any extraneous rays from the microscope lamp. These simple precautions are absolutely essential for critical study and should not be slighted. There is also reason to believe

⁸ May be obtained from Cogit, Boulevard St-Michel, 36, Paris-VIe, France, at about \$5.00 per 100 grams. I do not know of any American dealer who carries this stain in stock.

that it might be well to support the cover-slip so that the living cells will not bear its weight too directly.

Staining may be accomplished either by removing the tissue desired from the animal and placing it in a small dish of staining solution, or the stain may be injected in various ways into the living animal, or even given to it like food by way of the alimentary canal. The best procedure can be determined only by trial, and the correct dilution, or quantity, of the dye must be similarly arrived at. The following specific examples will indicate the general direction to be followed. Reference may also be made to part I, page 307, for further notes on the technical application of vital dyes.

Application of neutral red to animal tissues⁹

Accoyer ('24) removes the choroid epithelium (rat) and places it in a drop of physiological serum, to which a little 1 per cent neutral red is added (and also 0.1 per cent Janus green if a double stain (vacuome and chondriome) is desired). The stain occurs in about thirty seconds. Or he injects the staining solution and kills after about ten minutes (Janus green is not successful by this method).

Parat and his coworkers give practically no statement of method, but it is clear that they have placed the desired tissues in various dilutions of the stock neutral-red solution or have used the method of injection. Mitochondrial formations may be stained postvitaly with Janus green. (For example, Parat and Painlevé, '25 a.)

Avel ('25 a) allows a small drop of staining solution to dry on a cover-slip and then inverts it over teased-out cells, allowing the stain to dissolve slowly. (Owing to the prolonged time usually needed for a satisfactory neutral-red result, this method is not to be recommended for routine work

⁹ A curious case is reported by Bensley ('11), who applied neutral-red staining to the islet cells in the guinea-pig's pancreas. He observed the staining of many neutral-red granules which helped to bring out the outlines of 'Holmgren's canals' (Golgi apparatus), which appeared as colorless spaces among the deeply stained granules. This observation fails in a most unequivocal manner to agree with the claims of the vacuome theory.

with this particular dye, but might be useful for applying Janus green as a counterstain.) For vital staining on Amphibia, he injects 1 cc. of neutral red saturated in Ringer, killing the animals after various intervals. For tadpoles, he kept them alive in ordinary water solutions of the dye (great dilutions—up to 1:1,000,000), the animals always dying rather quickly (a few days usually).

Battacharya ('27), in the case of *Ascidia* and *Amphibia*, recommends injections into the epidermis and intraperitoneally of 8 to 12 cc., or the neutral red may be fed through the mouth apparently without injury to the animal. Ovary is well stained after a few days. For general staining purposes he uses neutral red one part in 500 parts of 0.6 per cent saline solution, keeping at 38° for twelve hours before use. Before using, dilute with equal parts of sea-water or Janus green of a strength similar to the neutral red.

Voïnov ('27) removes the gonads of *Notonecta* aseptically and places them in sterilized Ringer feebly colored with neutral red. Coloration of the vacuome begins in about an hour, but it is better to wait until time has been allowed for the concentration of the dye and the demonstration of all elements of the vacuome. Usually two to three hours is sufficient, and too prolonged a stain leads to artifacts. If such tissue be kept under aseptic conditions, in the dark, and at a relatively low temperature, the cells are perfectly preserved for study for twenty-four to forty-eight hours.

Hibbard ('28), working on the ovary of *Discoglossus*, finds it impossible to stain the developing eggs directly even in saturated neutral red. She was obliged to use the method of injection, using saturated neutral red in Ringer and injecting 2 to 4 cc. alternately into the lymph sacs, peritoneal cavity, and the digestive tract (by way of the mouth) every day for ten days. Then tease out the ovary in Ringer and examine.

Hirschler ('28) uses on fresh cells neutral red made up in 0.8 per cent saline in proportions of 1:4000 to 1:10,000. For double staining he uses:

Neutral red, 1:4000,	2 parts
Janus green	} 1:10,000, 1 part
or	
Gentian violet	

In the case of tissue cultures, the neutral red is added directly to the culture medium in proportions of from 1:50,000 to 1:100,000 as experience dictates (Lewis and Robertson, '16; Zweibaum and Elkner, '26 a, b). Policard ('26) places a small drop of neutral red (1:1000) directly on the culture.

From these notes and experience in this laboratory the following routine seems to be indicated:

1. For fresh tissue, dissect out the necessary part with as little injury and handling as possible. Undoubtedly, aseptic precautions would be desirable, but for general purposes this would not be considered necessary to good technic. Place the tissue in a small dish containing the stain. Use some normal fluid to which enough stock solution of neutral red is added to give a noticeable color—the exact shade must be learned by trial and experience. After an hour or two, tease out the material on a slide in a drop of the same normal fluid, cover, and study. As long as the cells are alive and in good condition, neutral red will, in general, stain only watery vacuoles, but after death the nucleus and other structures take the stain. As soon, therefore, as the cytoplasm or nucleus begins to show a trace of pink, the preparation should be discarded.

2. In case the staining is to be accomplished by injection, use much stronger solutions of the dye (up to full saturation), and repeat the injection several times over periods of several days. Only trial can give the exact details for a particular case. Finally, dissect out the desired part and tease in a normal fluid as before, for examination.

Such preparations are always short-lived, and their study must accordingly be completed at once without possibility of later recourse for comparison or critical reexamination. This is admittedly a very serious disadvantage, and has led to an effort to develop some procedure by which such preparations could be fixed and permanently mounted without losing the neutral-red stain. Several methods have now been described, but in all of them there is a considerable loss of the stain and a spreading of the coloration outside of the vacuoles. In

no case, therefore, has a really satisfactory result been yet attained.

Parat and Painlevé ('25 b) stain in neutral red, then fix in Turchini's fluid, Lugol, or by the Lewis method. In the first case, follow with a brief washing in water; in the other two, with a washing in potassium iodide to remove the excess of iodine. The Lewis method (Lewis, '22) has been much used, and a complete description is given in part I, page 315. The liquid of Turchini was developed to fix tissue and at the same time precipitate a vital dye previously applied. In this particular case, Turchini ('22) had in mind methylene blue, but the fluid is now recommended for neutral red. Turchini ('22) himself used Altmann's osmic-bichromate mixture for fixation of neutral red. The so-called liquid of Turchini ('22) is as follows:

Picric acid, saturated aqueous solution,	60 cc.
Formol,	20 cc.
Ammonium molybdate, ¹⁰ saturated aqueous solution,	20 cc.

This works well for Batrachia, but on warm-blooded vertebrates he used:

Picric acid, saturated aqueous solution,	200 cc.
2 per cent Osmic acid,	25 cc.

After proper fixation by any of these methods and washing, dry and mount finally in thick gum arabic (iodized). Tretjakoff ('28) mounts in glycerol or levulose.

Some method for properly fixing neutral red so that satisfactory permanent preparations can be made is urgently needed. I would suggest the possibility of developing methods such as that described by McJunkin ('25), Cash ('26), and Gardner ('27).¹¹ Gardner's technic is the most completely worked out, and consists in fixation, after vital staining with neutral red, in alkaline Zenker-formol. The subsequent treatment is designed to avoid as much as pos-

¹⁰ Zawarzin ('09), after staining *intra vitam* with neutral red, fixed the dye with ammonium molybdate. Faris ('24) (refer also to Kordes, '24) has recently used phosphomolybdic acid and phosphotungstic acid, but preferably ammonium tungstate as a mordant for subsequent staining with neutral red. This method is used after fixation in Bouin and is obviously not at all applicable to *intra-vitam* preparations. But the mordanting fluids which he suggests might be developed into something useful for color fixation under vital-staining conditions.

¹¹ See also the procedure suggested by Scott ('28) for blood cells, although the method seems of doubtful applicability to the vacuome problem in general.

sible contacts with alcohol and particularly water, which are very injurious to neutral-red staining. This method has not been tried in connection with definite work on the vacuome, and I will therefore not now give it in detail, but there is one suggestion given by Gardner which seems to me particularly worthy of note. He points out that if fixation progresses too rapidly, the dye will be dissolved, but that this may be avoided by placing the stained tissue in a parchment sac full of neutral-red solution and suspending the sac in a jar of fixative. After thirty to forty minutes, the dye in the sac is precipitated, and the tissues are then removed from the sac and placed directly in the fixative. The general fixation suffers, but the vital stain is well preserved.

Application of neutral red to plant tissues

Neutral red has been extensively employed by Guilliermond and others in the study of plant vacuoles, more particularly those of meristem cells and of simple forms like fungi and molds. In some cases the parts may be stained and studied directly with the greatest ease (molds, for example), but in other cases before or after staining the cells must be more or less teased apart for study. In general, the application of neutral red to cells of the higher plants is difficult or impossible, because of the heavy cell walls which are not readily penetrated by the dye. Furthermore, the stain works best on the meristem cells, and after the large vacuole, characteristic of the differentiated plant cell, has been developed, neutral red usually stains coagulated (?) masses of vacuolar contents ('endochromidies' of P. A. Dangeard) rather than the vacuole as a whole (see, for example, papers by Guilliermond).

Workers on plant material have given fewer details for the use of neutral red than in the case of animal material. Guilliermond ('27) simply states that, in using vital stains for the vacuome, the concentration of the staining solution must be varied according to the material—there is no rule. The neutral-red solution should probably be made up as

already indicated on page 250. P. Dangeard ('23 a), observing that neutral-red solutions made up in advance often precipitate (but see p. 250 on this point), finds it practically convenient to add a little of the solid dye to water just before using, the amount being roughly determined by previous experience (usually concentrations of 1:1000 to 1:10,000; the most favorable being about 1:5000). The cells may be mounted for observation in isotonic sugar solutions (Guilliermond, '20) if desired, or in juices freshly expressed from similar material (Bensley, '10). No data for the preservation of such preparations specifically applicable to plant material are yet available.

Miscellaneous vital dyes

The most favorable vital dye for the vacuome is undoubtedly neutral red, but many others have been used with more or less success, particularly cresyl blue on plant material.

On *animal material* the following data on the vacuome are available:

Janus green—Parat and Painlevé ('25 c), but negative according to Karpova ('25). The preponderance of evidence, however, indicates that Janus green will stain vacuoles slightly in time, but the method is not recommended.

Nile-blue sulphate (and *chloride*), *new methyl blue*, *methylene blue*, *toluidin blue*, and *brilliant cresyl blue*, all yield positive results on *Helix* sperm cells, according to Karpova ('25). On the same material Lee ('96) long ago obtained positive staining with *methylene red* (released by 'maturation' of a solution of methylene blue).

Policard ('26) mentions the use of *tropeolin OO*, *bromphenol blue*, and *bromcresyl purple*; and Zweibaum and Elkner ('26 a) have used *Bismarck brown*.

In no case is the result with vital dyes other than neutral red sufficiently successful to make their use worth while.

On *plant material*,¹² dyes other than neutral red are frequently quite successful, for example:

¹² The references here are by no means exhaustive, but indicate fully the nature of the dyes which can be used.

Janus green—P. A. Dangeard ('16 b), P. Dangeard ('23 a), Guilliermond ('27). The last states that Janus green stains more slowly and less regularly than neutral red. By combining the two dyes, a simultaneous stain of chondriome and vacuome can be secured, as already noted for animal material. If the Janus green penetrates poorly, violet dahlia may be substituted. This dye also may stain the vacuome as in the case of Janus green.

Cresyl blue—P. A. Dangeard ('16 a, b, c), P. Dangeard ('23 a), Puymaly ('24), and Guilliermond ('27). The last-mentioned author states that cresyl blue and toluidin blue give results equal to neutral red, but penetrate less easily.

Nile blue—Guilliermond ('20 and '27); penetrates easily, but is very toxic.

Methylene blue—P. A. Dangeard ('16 a, c), P. Dangeard ('23 a), and Guilliermond ('27). Guilliermond states that this is the poorest penetrator of all the vital dyes.

P. Dangeard ('23 a) uses cresyl blue in the same concentrations as neutral red (p. 256); methylene blue and Janus green in a concentration of 1:100,000.

It should finally be noted that the ideal condition for studying the vacuome would be one in which the vacuolar contents are stained by some natural coloring matter. This result is occasionally attained through the formation of the red anthocyanin pigments. The possibilities thus to be taken advantage of were first realized by Guilliermond ('13), whose idea was subsequently adopted by Pensa ('13) with most interesting results. Further search for such natural vital staining is much to be desired.

Methods of fixation and staining for permanent mounts

Permanent preparations in which the vacuome is sharply differentiated by methods of staining or metallic impregnation are in many respects more desirable for critical study than the transitory preparations of intra-vitam methods. Such methods are, in point of fact, now available, but more particularly for plant material, as indicated in the following résumé.

In *animal tissues* the vacuome is rarely demonstrable by any known method for permanent mounts. In tissue culture

Zweibaum and Elkner ('26 a) once obtained a complete blackening of the vacuome with the Da Fano method, but silver methods in general are not successful. Hirschler's ('27 b) new method sometimes gives very beautiful positive stains of the vacuome (part IV, p. 380).

Parat and Bergeot ('25) find that the vacuome, as is usually to be expected, gives negative results with sudan III, scharlach R, and indophenol blue applied to frozen sections after formol fixation. Similar negative results are yielded by Ciaccio and by Dietrich (see also part V). Nevertheless, by the last-named method, the vacuoles are brought out as clear droplets on a gray-blue background—a result similar to that obtained by Zweibaum and Elkner ('26 a) with various Golgi-apparatus methods. This behavior has led Parat ('26) to the development of a new method, as follows:

Parat's modification of Smith-Dietrich (Parat, '26):

1. Fix in Zenker-formol (Helly) for two to six hours, according to the size of the pieces.
2. Postchrome for forty-eight hours in saturated potassium bichromate at 40°C.
3. Wash in running water for twelve to eighteen hours.
4. Then embed as usual in paraffin (or cut frozen sections).
5. Stain by Kull's method; or, without mordanting, in Kultschitzky's hematoxylin differentiating in borax-ferricyanide as in the original Smith-Dietrich method (part V, p. 111); or stain in warm sudan III for some hours as in Ciaccio's method (part V, p. 109). Frozen sections give the best microchemical results.

Result. The chondriosomes are stained dark on a blue-black background, against which the unstained vacuome stands out as clear droplets. These vacuoles correspond exactly with those stained intravita with neutral red. Many advantages are claimed for this method, for which see the original paper.

Bhattacharya ('27) for this method advises fixation for twenty-four hours in Helly, or Regaud's formol-bichromate. He gives the necessary staining formula as:

Hematoxylin (dissolved in a little alcohol),	-	1 gram
2 per cent Acetic acid in water,		100 cc.

In *plant tissues* the vacuome can be demonstrated by positive staining in many kinds of cells and by many different

methods. In no case are such methods specific, however, and their successful use seems to depend on the chemical nature of the vacuolar contents in particular cases. The Golgi-apparatus methods as applied to animal tissues sometimes demonstrate the plant vacuome, sometimes they do not; and in practically all cases the results on meristem tissues are not duplicated in more differentiated types of cells. Mitochondrial methods also are sometimes successful, especially in case phenolic or anthocyanic materials are present in the vacuoles (Guilliermond, '21). In any case, only methods which yield a positive result through the direct staining of the vacuoles, are useful, since negative images in meristem cells are impossible to decipher in most cases.

Positive results may thus be obtained on occasion after a great variety of technics, as follows:

Silver impregnation (part II)—Pensa ('10? and '17); Laburu ('16) by Cajal's formol-uranium nitrate; Sanchez ('22 a, b, c) by Rio-Hortega's modifications of Achúcarro's method, also methods of Cajal and Da Fano; Guilliermond and Mangenot ('22) by methods of Golgi, Cajal, and Da Fano, etc.

Osmic impregnation (part III)—P. A. Dangeard ('16 b) with Sjövall's method. Guilliermond ('26 a); Bowen ('26, '27 b) by methods of Kolatchev, Hirschler, and Weigl; Guilliermond ('26 b) by Kolatchev; Emberger ('23), and P. Dangeard ('23 a).

Miscellaneous methods (part IV)—P. A. Dangeard ('16 b) with Altmann's method. I have also found this (Kull's modification) to yield occasional results, especially after Champy fixation. I have also found the Benda stain successful, sometimes remarkably so, after chrome-osmic fixation. For much further information¹³ on the demonstration of the vacuome, see particularly P. Dangeard ('23 a) (and also Emberger ('23) and Guilliermond's many papers,¹⁴ particularly that of 1927). In all cases however, these miscellaneous

¹³ See also page 265 for Bensley's methods and interpretation.

¹⁴ For bibliography see Bowen, '27 a.

methods yield sporadic results at best and are in no sense to be recommended for routine study of the plant vacuome. The demonstration of the plant vacuome has been particularly studied by Bowen ('26, '27 b, and '28) and Guilliermond ('27), who have given very extensive technical notes on impregnation methods. Guilliermond has worked particularly with the Kolatchev osmic method and the silver methods of Cajal and Da Fano. Of these he finds the silver methods much preferable as being more certain and more apt to impregnate only the vacuome. Nevertheless, the silver technic is inconstant and may also blacken the chondriome, either alone or in conjunction with the vacuome. Frozen sections are better than the paraffin method. The Kolatchev method, on the other hand, succeeds well with fungi, but on higher plants is even more inconstant than the silver methods. Guilliermond finds, further, that it usually blackens both chondriome and vacuome, and that attempts to decolorize the chondriome alone do not usually succeed. My own experience has been that the osmic methods of Kolatchev and Weigl are very useful and yield excellent results on certain plants. In other cases they are almost, if not entirely, unsuccessful. The explanation of this behavior is not clear, though the chemical constitution of the vacuolar contents seems to me the deciding factor. At best these methods are erratic, but if proper material can be found, they will on occasion yield remarkably clear-cut and beautiful results. In both silver and osmic preparations the vacuoles are more or less completely blackened, the exact type of impregnation being subject to several variations.

Guilliermond ('27) uses the Cajal method substantially as given in part II, page 97. He fixes twelve hours in the non-alcoholic mixture, using 100 cc. of distilled water instead of the 85 cc. given by Cajal in 1914; followed by 1.5 to 2 per cent silver nitrate for thirty-six to forty-eight hours. Reduce, etc., as usual. Tone sections in 2 per cent gold chloride if the impregnation will stand it, and fix in saturated sodium hypophosphite. Counterstain, if desired, in erythrosin.

Guilliermond ('27) uses the Kolatchev method essentially as I have given it in part III, page 257. He fixes in Champy (the 7:7:4 formula) on ice, and, after washing as usual, osmicates in 2 per cent osmic acid for eight to fifteen days at 40°C. Sections made from such material are usually completely blackened, and must be bleached by the permanganate-oxalic method (part III, p. 266). Counterstain in Kull, if desired. My own method of using Kolatchev will be noted more fully on page 269, but in connection with the vacuome it may be observed that osmication for so long a period at 40°C. would not be favorable treatment for animal tissues in which the Golgi apparatus was to be demonstrated. It seems to me preferable, where possible, to osmicate for shorter periods, three to eight days, at a temperature of 35°C., with or without an initial incubation for eight hours at 40°C. (p. 269, and part III, p. 257). In certain cases I have found that the Weigl method is very much superior to the Kolatchev method as applied to the vacuome, the preparations having a remarkable wealth of detail and the sharpest contrast between vacuoles and cytoplasmic background. The method employed is exactly as given on page 250 of part III. This corrosive-osmic fixative has also a remarkably selective blackening effect on the intravacuolar coagulation masses often shown, for example, in Guilliermond's figures from preparations by Regaud and similar methods. For this purpose osmication is not required, and it is necessary only to fix for twenty-four hours and then wash and embed as usual.

The vacuome in Protozoa

The vacuome in Protozoa has been only recently investigated,¹⁵ with results which indicate its occurrence just as in other cells, both animal and plant. Grassé ('25) finds the

¹⁵ But many older observations and some more recent ones have been made on vacuolar structures (not including contractile vacuoles) in the Protozoa, without reference to the particular development of the matter here under discussion. See, for example, Fauré-Fremiet ('09), Edwards ('24), etc. These observations will eventually have to be reexamined in the light of modern conceptions of the vacuome.

vacuome in *Euglena* blackened by Cajal's method and later ('26 b) reports a complete impregnation by osmic acid, especially after Champy fixation. Most of the results, however, are from intra-vitam study with neutral red or cresyl blue, used as already noted. This was first accomplished by P. Dangeard in 1923 b in *Ceratium* and *Peridinium* with neutral red. Later (1924), the same worker got similar results with neutral red and cresyl blue on *Phacus*, *Trachelomonas*, and several species of *Euglena*. P. A. and P. Dangeard ('24) report similarly after the use of the same dyes on *Chlamydomonas ovata*, *C. reticulata*, *Chlorogonium euehlorum*, *Gonium sociale*, *Eudorina elegans*, and *Volvox*. Grassé ('25) reports results on *Euglena* with neutral red and cresyl blue similar to those of P. Dangeard. Grassé ('26 a) also reports staining of certain vacuoles in *Tetramastix* with Janus green. In all cases the vacuome consists of small, scattered, spherical vacuoles, like those in animal cells generally. Networks such as often occur in plant cells have not yet been observed. The meaning of the protozoan vacuome is entirely obscure, but it is worthy of note that no workers on the Golgi material in Protozoa have attempted to homologize these vacuoles with the Golgi apparatus. In this connection it should be noted that the contractile vacuoles of Protozoa do not stain intra vitam as do the other vacuoles and have not thus far been considered as a part of the vacuome proper.

The excretion of vital dyes

Following the work of Möllendorff, who had shown the relation between intracellular accumulations of vital dyes and the formation of certain kinds of excretion vacuoles, the relation of such vacuoles to the Golgi apparatus has been studied by several workers with most suggestive results (Jasswoin, '25; Nassonov, '26, '27; Glasunow, '28; etc.). A most interesting outgrowth of such experiments has recently been published by Chlopin ('25 and '27), particularly interesting in the present connection, because he made use of neutral red as an injection dye. In consequence of neutral-red injec-

tions, he finds structures appearing in the cell to which he gives the name of 'krinome.' The krinome is to be taken as the symbol of certain secretory processes called forth by the presence of the vital dye, and is characterized by a decided basophilia in fixed preparations. Frequently, also, it assumes marked topographical similarities to the Golgi apparatus. The similarity of such appearances to the results of Jasswoin, Nassonov, and others, already mentioned, is very striking, although given a somewhat different interpretation by Chlopin. But in the present connection the most astonishing fact is that the krinome is not demonstrated in the final preparations by the retention of the original neutral-red stain, but by the addition of a histological stain. Thus, Chlopin ('27, which see for extended technical details) fixes in Zenker-formol (also Champy or 10 per cent formol), and stains in basic nuclear dyes, such as thionin, methylene blue, Fe-hematoxylin, etc., the most favorable being thionin-aurantia and especially eosin-azure.

The relation of such results to Parat's vacuome studies at once raises many points difficult to interpret, and leaves one much in doubt as to the validity of the use of neutral-red injections in studying the vacuome proper. The whole problem must be left unanswered for the present and suggests many interesting points for further research.

Critique of the methods for demonstrating the vacuome

From this résumé of the technic for demonstrating the vacuome, two facts emerge very clearly. The first of these is that the comparison of the vacuoles with the classical Golgi apparatus seems a most questionable point of departure for the study of the vacuome. The vacuole system presents so many points of interest for its own sake that it would seem the part of wisdom to learn first about the vacuoles as such, and avoid a too insistent emphasis of its possible homologies with the Golgi apparatus. The second fact is the clear necessity of some method which can be used with reasonable certainty for the exact demonstration of the vacuome in permanent mounts.

With respect to the available methods, that of vital staining with neutral red is just now by far the best one, especially in animal tissues. Better methods of fixing the stain for permanent mounts are just now most urgently needed, and if found will go a long way toward supplying an adequate technical weapon. In plants the methods of silver and osmium impregnation offer obviously the best possibilities and should certainly be given a trial, although in many (most?) cases satisfactory results are not to be expected.

I would suggest that anyone interested in the vacuome problem begin by preparing the neutral red (Microcolor brand) solution as described on page 250, and then apply it in various ways to a large range of material. In this way experience and discrimination can be attained—remembering always that neutral-red results are valid only when the nucleus and cytoplasmic background are absolutely devoid of the faintest tinge of red. From such a foundation an attack may be made on the matter of neutral-red fixation in permanent mounts, or the development of impregnation methods of more general application, and then the many other problems which now cluster around intracellular vacuoles of all kinds in both plant and animal tissues.

METHODS FOR PLANT MATERIAL

I come now, in conclusion, to a matter which, of all the unsolved problems connected with the Golgi apparatus, is perhaps at once the most annoying and the most inexplicable. This is the question as to what constitutes the homologue of the Golgi apparatus in plant cells. Into the various opinions which have been and still are held on this question, it is here impossible to go in any critical way. I propose only to deal with the methods which have been used for demonstrating something which the author has identified as Golgi material. In a concluding critical paragraph, a few comments will be made on the validity of the identifications.

The vacuome as Golgi apparatus

As just given in detail in the preceding section on the vacuome, it has been particularly the contention of Guilliermond ('27, for example) that plant vacuoles are the plant Golgi apparatus. Guilliermond's contributions to technic have been given in connection with the preceding vacuome section, but some additional notes are here collected from the work of other authors who have described as the Golgi apparatus what is obviously the vacuole system.

The earliest definite contribution to this problem is a paper by Bensley ('10), whose discussion of the nature of the vacuolar system in plant cells is really the point of departure for Guilliermond's recent interpretations. Bensley suggested that the vacuolar canal system in meristem cells may actually be the homologue of Holmgren's canalicular apparatus in animal cells. In what would now be a more acceptable terminology, this is tantamount to saying that the plant vacuome is equivalent to the animal Golgi apparatus. Bensley's observations were made primarily on fixed material, but he checked his results by intra-vitam examination of plant cells mounted in the freshly expressed juices of similar tissues. He recommends for permanent preparations the following:

Fix in:

1. Kopsch's fluid.

2.5 per cent Potassium bichromate,	75 cc.
Neutral formalin,	25 cc.

Or 2. Formalin-bichromate-sublimate.

Neutral formalin (freshly distilled),	10 cc.
Water,	90 cc.
Potassium bichromate,	2.5 grams
Mercuric chloride,	5 grams

In either case stain with Fe-hematoxylin or Flemming's triple stain.

Guilliermond ('27) gives much more complete details for the second method, as follows:

1. Fix for twenty-four hours in the formalin-bichromate-sublimate mixture as given above.
2. Wash for twenty-four hours and then treat as usual with iodine, to eliminate the sublimate. Wash again in water for five minutes.
3. Place in a saturated aqueous solution of copper acetate for five to ten minutes.
4. After washing about one minute, stain for five to ten minutes in 0.5 per cent hematoxylin.
5. Wash and treat with a neutral aqueous solution of 5 per cent potassium bichromate, until the material blackens (about thirty seconds).
6. Embed in paraffin and section.
7. Differentiate in Weigert's ferricyanide solution, and complete the preparation as usual.

Result. The vacuolar canals are clear on a gray background. Similar results are obtained with Regaud.

At about the same time, Pensa ('10) observed what was probably the vacuome in a plant cell after the direct application of silver reduction¹⁶ to fresh sections of the ovary of *Lilium*. He considered the relation of such structures to the Golgi apparatus, but left the matter entirely open. Further notes on the same problem involving the use of other methods are also given particularly in Pensa's paper of 1917. Laburu ('16) later demonstrated, in *Solanum*, structures now clearly to be considered vacuome, but which he merely identified as Golgi apparatus without further comment. He fixed pieces of year-old potato for twenty-four hours in Cajal's formol-uranium nitrate (part II, p. 97), followed by silvering for twenty-four hours at room temperature in 1 per cent silver nitrate and then the usual reduction for twenty-four hours. After washing, he cut sections free-hand for final examination in balsam. Further studies on the Golgi apparatus in plant cells, which appear likewise to have been primarily demonstrations of the vacuome, have been made by Sanchez ('22 a, b, c) and Luelmo ('23). Sanchez used the silver methods of Cajal and Da Fano, and particularly Rio-Hortega's modifications of Achúcarro's method. He gives no technical details, but the usual procedure in all these

¹⁶ For details of the method, see Pensa ('12).

methods has been given in part II. Luelmo used Drew's method, which will be found on page 267.

The plastidome as Golgi apparatus

In two cases workers have identified as the probable homologue of the Golgi apparatus in plant cells structures which pretty certainly belong to the plastidome—formed elements, in other words, which are, or give rise to, plastids in differentiated plant cells and have often been considered, though very possibly incorrectly, as chondriosomes. Thus Smirnow ('06) found bodies in the hyacinth root tip, which he likened to Golgi apparatus, in Flemming-hematoxylin and other preparations, but obtained constantly negative results (after two- to thirty-day periods) with the Kopsch method (part III, p. 245). Drew ('20), working on more differentiated cells in the onion root, developed a special technic for demonstrating bodies which are probably leucoplasts, but which he classed as Golgi bodies.

Drew's method (Drew, '20):

1. Fix for twenty-four hours, preferably at 37°C., in:

Formol,	20 cc.
Cobalt nitrate,	2 grams
Sodium chloride,	0.8 gram
Water to make,	100 cc.

2. Cut frozen sections after soaking an hour in gum syrup, and fix the sections, after washing in water, on gelatin-coated slides with formalin.

3. Rinse in water to remove the excess of formalin and mordant for fifteen minutes to one hour or longer at 50° to 55°C. in equal parts of 4 per cent chromic acid and 2 per cent osmic acid.

4. Rinse in water and mordant further with 3 per cent Fe-alum for fifteen minutes.

5. Stain in 0.5 per cent hematoxylin for fifteen minutes at 50°C.

6. Differentiate in 3 per cent Fe-alum, cold, until the nuclei are pale brown.

7. Transfer to 2 per cent pyridin for two minutes.

8. Wash in running water two to five minutes and mount as usual in balsam.

Result. Two types of bodies are stained. One consists of many small scattered granules, identified as chondriosomes; the other, of larger bodies identified as Golgi apparatus. These types are probably the two categories of chondriosome-like bodies well known in plant cytoplasm (refer for further details to Bowen, '27 a, b), and very certainly have no relation to the Golgi apparatus. When chromated for short periods (step 3) only the chondriosome granules are visible, while with longer periods the presumed 'Golgi bodies' (probably leucoplasts) are more conspicuous. As already noted (p. 267), Luelmo ('23) has used this method, apparently demonstrating the vacuome.

The osmiophilic platelets as Golgi apparatus

It is a rather curious fact that exactly those methods which are proving to be best for demonstrating the Golgi apparatus in animal cells, viz., the methods of osmic impregnation, should not have been seriously applied to plant tissue until 1918.¹⁷ In that year the Kolatchev method was tried on root tips by Nassonov ('18) with most interesting results. Subsequently, I applied the same method and also the methods of Weigl and Hirschler to many kinds of plant cells (Bowen, '26 a, '27 a and b, and '28 a), and at about the same time Guilliermond ('26 b and '27) reported his independent results, especially with the Kolatchev method. It is now clear, as I have pointed out elsewhere in detail ('28 a), that the method of Kolatchev in particular is not nearly so specific in plant tissue as in animal. Whereas, in the latter, only the Golgi apparatus is regularly blackened, in plants almost any structure in the cell may be impregnated. Among the bodies which are thus on occasion brought to view are all the categories of cytoplasmic elements heretofore described by botanists (except probably the central apparatus). But in addition I have found another and entirely new class of bodies to which the descriptive name of osmiophilic platelets

¹⁷ Except the Kopsch method, with which Smirnow ('06) got only negative results, and the Sjövall method used by Guilliermond ('12) and P. A. Dangeard ('16 b) apparently without any results considered worth while following up.

has been tentatively applied. These bodies are shaped apparently like a circular plate or disc, the periphery of which appears intensely blackened by proper treatment with osmic acid. Of all the constituents in the plant cell which can be blackened by osmic impregnation, these platelets are by all means most frequently and satisfactorily demonstrated. In other words, the method is partially specific for the osmiophilic platelets, though often yielding additional results on other cellular components. I have adduced evidence to show that these bodies are the probable homologue of the animal Golgi apparatus (Bowen, '27 b). This view, indeed, the reality of the structures described, has been violently attacked by Guilliermond ('28), who holds that the platelets are merely artifacts produced from other and well-known cytoplasmic bodies. It is difficult for me to see how such an unqualified position can be taken by any one who has seen adequate preparations of the platelets, for they can be demonstrated either alone or simultaneously with the plastidome and 'inactive chondriome,' and there is not the slightest evidence that the platelets arise through deformation of other formed bodies. However, my findings have recently been examined by Gatenby, whose students have repeated my technic, and he in every way supports my contention that the osmiophilic platelets are probably the plant Golgi apparatus.

These platelets may be demonstrated by the methods either of Weigl, Kolatchev, or Hirschler (part III). In the case of Weigl, the procedure is followed exactly as given on page 250 of part III, the fixation time being taken as one to four hours. This method is not nearly so satisfactory for the platelets as the Kolatchev technic. I have given my procedure with this latter method very completely in a recent paper (Bowen, '28 a), and it will be sufficient here merely to refer to the almost equally complete details given in part III, p. 257. Fix in the original Champy formula (7:7:4) given as formula I in part III, page 257. Follow the directions exactly as given, and in step 3 divide the material into four lots and osmicate by the four possible combinations described. If

time and other facilities are not sufficient, the best single method is probably that of osmicating in 2 per cent osmic acid first at 40°C. for eight hours, and then at 35°C. for a total time of three or four to eight or nine days. Complete the technical operations exactly as given, cutting sections not more than 5 μ thick.

The appearance of the preparations can often be greatly enhanced by bleaching (part III, p. 264). I have tried several methods, but the permanganate-oxalic technic yields perhaps the best results. The complete details are given on page 267 of part III. Finally, if desired, the preparations can be counterstained. I have found that the best method is to bleach as far as is safely possible and then use the Kull technic as described in detail in part III, page 272.

With this method the rims of the osmiophilic platelets are demonstrated in intense black on a yellowish background, which may sometimes be made almost glass-like by bleaching. After Kull staining, the chondriome or plastidome or both together may be stained in red, with the platelets as black rings. The center of the platelets is almost invisible, being sometimes slightly blackened or browned, and in the greatest contrast to the intense black of the rim.

Supplementary notes. Gatenby ('28) and two of his students have recently reported the successful use of the Kolatchev method, as above outlined, on plant material, where they find that it never fails to demonstrate the osmiophilic platelets if properly carried out. They slit up root tips before fixation, for which they employ Champy or Nassonov's ('24) modification number II, given on page 240. Osmication is carried out in 2 per cent osmic acid at 30° to 35°C. for three to seven days, four days being usually sufficient.

Also to be noted is the paper of Joyet-Lavergne ('27), who treated pollen grains with osmic acid of 0.2 to 2 per cent strength. The action is inspected occasionally and checked when desired. In this way he demonstrates blackened arcs or granules constituting the border of small vacuoles, which in turn can be stained *intra vitam* by neutral red. He thinks

the blackened elements are the Golgi apparatus, but their morphology and technical behavior does not seem to indicate a homology with the true osmiophilic platelets.

Critique and suggestions for practice

I think it fair to say that at the present time, of the known structural elements in plant cytoplasm, the only possible homologues of the Golgi apparatus are the vacuome and osmiophilic platelets. Of these two I must confess a very strong personal bias against the identification of the vacuole system as the equivalent of the Golgi apparatus in animal cells (Bowen, '27 a). Nevertheless, the relation of certain types of vacuoles to the animal Golgi apparatus (acrosomal vesicle in the spermatid, for example) is beyond doubt, and the possibility that the plant vacuoles may have some unrecognized connection with the Golgi apparatus in plant cells cannot as yet be excluded. Such possibilities suggest the desirability of pushing the study of the vacuome further by the use of the methods already noted. But of even more immediate interest is the extension of our knowledge of the osmiophilic platelets and the gaining of some generally accepted opinion as to their nature and interpretation. In this direction there seems open a most attractive field for investigation. The technical treatment of plant cells is difficult, but the time is now ripe for a really serious attack upon the whole problem of plant cytoplasm, and in this connection some well-established conclusions as to the nature of the Golgi apparatus are of the first importance.

For those who may be interested in the osmiophilic platelets, I would suggest making some initial trials with the lateral roots of *Vicia Faba*. The seeds should be soaked for a day in water and planted in earth. When the plants are several inches tall, wash the earth from the root under gently running water and cut off the tips of such lateral roots as have not reached a length of more than 1 to 1½ inches. Follow the Kolatchev technic exactly as given on page 269, and results are practically certain to be satisfactory in some degree at least.

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THE ANATOMY OF THE FORE LIMB OF THE ALBINO RAT AT APPROXIMATELY THE TIME IN FETAL LIFE WHEN SOMATIC MOVEMENT BEGINS

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SIX FIGURES

To a student of morphology the question naturally arises as to the relation between the development of behavior and the anatomy of the animal. In a lower form, *Amblystoma*, the development of the behavior pattern has been completely worked out and close correlations have been made with the anatomical foundations of this development (Coghill, '09, '14), but no such work has been completed on a mammal. It is obvious that properly controlled experiments designed to analyze the movements of human embryos in utero are impossible, although restricted observations upon early human fetuses obtained at operation have been recorded (Minkowski, '20, '21). Therefore, the question arises as to which laboratory animal is best adapted to meet the requirements of this problem. Tilney and Casamajor ('24) have studied behavioral relations of kittens as related to the structure of the central nervous system, and Weed and Langworthy ('26) and Langworthy ('27) have made correlated physiological and morphological studies of the cerebral cortex of kittens and opossums. We have chosen the domesticated rat for reasons best expressed by Donaldson:

If the life span of three years in the rat is taken as equivalent to ninety years in man, it is found that the growth changes in the nervous system occur within the same fraction of the life span (i.e., at the equivalent ages) in the two forms. What is true for the

nervous system is also probably true for some of the other systems and this makes possible the cross reference of the results from the two species, with a high degree of precision.

Lane has made tests upon fetal as well as upon young rats, in studying the development of the function of the special senses in correlation with the structure of the nervous tissues concerned. Although his observations allowed him to determine a stage of development when movement appeared in response to tactile stimuli, his method required the removal of the uterus from the body of the mother with resultant early asphyxia of the fetus, so that a given experiment was of brief duration and subject, like Minkowski's, to abnormal conditions. Swenson ('25) has done much work upon living fetuses of the albino rat in order to determine the extent of movements. His method consists of the induction of cerebral anemia whereby normal conditions are approximated. According to his protocols filed in The Wistar Institute (Swenson, '28 b), movement begins in the albino rat during the sixteenth day, i.e., more than two-thirds of the period of gestation. After this time, movements can be elicited in the trunk and limbs as the rats develop progressively through the 'bilateral' stage to the rotation stage (Swenson, '28 a).

Since bodily movements of vertebrate animals are due to contraction of muscles in response to stimuli, with the resulting angulation of one part on another, any attempt to analyze the development of this mechanism must deal with the following questions, as, for instance, whether the muscles of the non-motile rat have differentiated and have the ability to contract, whether the skeletal units are sufficiently rigid to serve as levers and to allow movement between units, whether the nerves have made contact with the muscles and are functional, and whether the connections of the central nervous system have been established. Angulo ('27) has described the motor-cell columns of the cervical cord of the newborn rat and he is making further studies upon the cervical cord in rat fetuses of different stages of development. Hogg ('28) has taken up the study of the motor-ganglion cells of the

brain. As a part of the same general problem, the writer has undertaken an investigation of the peripheral mechanism.

The present study has to do with the gross extent and connections of the muscles and skeleton and the relation of the nerve trunks to them. Following this, it is planned to study the condition of the structures cytologically, the aim of the investigation being to discover any possible correlation of structure with function. The material chosen was the 'non-motile' rat of about the age when muscular movements begin, and consists of the identical individuals that had been tested for movements and found negative. To avoid dispersion of energy in this investigation, observation has been confined to the region of the fore limb.

Serial sections of the fetuses were studied, following which wax reconstructions of the skeleton and of superficial and deep dissections of the muscles and related nerves were made of one fetus (Swenson's R 79 F 9) according to the method of Born as described by Bardeen ('01). A model of the cephalic two-thirds of the embryo was also made, with one side dissected to show the central nervous system. These models were made with an enlargement of fifty diameters, and by their aid the gross anatomy of the fetus can be directly visualized. The crown-rump length in Ringer's solution was 12 mm. There were four other 12-mm. fetuses of this litter. Four measured 11.5 mm. and were non-motile, while the largest member of the litter, measuring 13 mm., was motile.¹

The following description of the anatomy is based chiefly upon these reconstructions, but checked by means of detailed microscopic examination of the actual serial sections of the fetus reconstructed and of several other fetuses of the same age.

¹Dr. A. W. Angulo permits me to say that, following Swenson's method, he has obtained records of movement in the albino rat of 13.2 mm. at 379 hours (fifteen days nineteen hours) of gestation. The measurement was taken in Ringer's solution by means of fine-pointed calipers with vernier attachment and under a magnifier.

THE SKELETON

The skeleton at this age shows no bone formation except a faint center in the clavicle and a trace in the mandible, but the skeletal parts of the limbs, particularly in the proximal segments, are well represented by precartilagel, in the center of which some elements contain cartilage. The outlines of the separate parts are not entirely definite, but shade off into the mesenchymal perichondrium with which the attachments of the muscles are continuous.

ABBREVIATIONS FOR ALL FIGURES

<i>ac.</i> , acromion	<i>m.tri.</i> , m. triceps
<i>cl.</i> , clavicle	<i>n.ax.</i> , n. axillaris
<i>ext.m.</i> , extensor mass	<i>n.int.</i> , n. intercostalis, ramus lateralis
<i>fl.m.</i> , flexor mass	<i>n.lat.th.</i> , n. thoracalis anterior lateralis
<i>gang.</i> , spinal ganglion	<i>n.med.</i> , n. medianus
<i>hu.</i> , humerus	<i>n.med.th.</i> , n. thoracalis anterior medialis
<i>hu.h.</i> , head of humerus	<i>n.mus.ct.</i> , n. musculocutaneus
<i>m.ac.sc.</i> , m: atlantoscapularis	<i>n.rad.</i> , n. radialis
<i>m.bi.</i> , m. biceps	<i>n.sup.</i> , n. suprascapularis
<i>m.br.rad.</i> , m. brachioradialis	<i>n.sub.</i> , n. subscapularis
<i>m.del.</i> , m. deltoideus	<i>n.th.dr.</i> , n. thoracodorsalis
<i>m.epi.</i> , m. epitrochlearis	<i>n.th.l.</i> , n. thoracalis longus
<i>m.ext.cp.</i> , m. extensor carpi ulnaris	<i>n.tri.</i> , nerve to m. triceps
<i>m.fl.cp.</i> , m. flexor carpi radialis	<i>n.ul.</i> , n. ulnaris
<i>m.inf.</i> , m. infraspinatus	<i>nn.ul.med.</i> , ulnar-median trunk
<i>m.lat.d.</i> , m. latissimus dorsi	<i>per.</i> , pericardium
<i>m.lev.sc.</i> , m. levator scapulae	<i>pi.</i> , pisiform
<i>m.om.h.</i> , m. omohyoideus	<i>pl.cerv.</i> , cervical plexus
<i>m.oc.sc.</i> , m. occipitoscapularis	<i>pl.cerv.a.</i> , ascending branches of cervical plexus
<i>m.p.ma.</i> , m. pectoralis major	<i>r.III.</i> , third rib
<i>m.p.mi.</i> , m. pectoralis minor	<i>rad.</i> , radius
<i>m.rect.ab.</i> , m. rectus abdominis	<i>scap.</i> , scapula
<i>m.rh.</i> , mm. rhomboidei	<i>sp.</i> , spine of scapula
<i>m.s.sp.</i> , m. supraspinatus	<i>sp.cd.</i> , spinal cord
<i>m.ser.ant.</i> , m. serratus anterior	<i>str.</i> , sternum
<i>m.st.h.</i> , m. sternohyoideus	<i>td.ext.</i> , extensor tendon
<i>m.st.ma.</i> , m. sternocleidomastoideus	<i>td.fl.</i> , flexor tendon
<i>m.st.th.</i> , m. sternothyroideus	<i>tr.ul.med.</i> , ulnar-median trunk
<i>m.sub.</i> , m. subclavius	<i>ul.</i> , ulna
<i>m.t.ma.</i> , m. teres major	<i>vt.neu.</i> , neural process of vertebra
<i>m.t.mi.</i> , m. teres minor	<i>vt.tr.pr.</i> , transverse process of vertebra
<i>m.tr.</i> , m. trapezius	

The scapula is situated in a plane almost parallel with the median plane of the body, and the vertebral border is nearly parallel with, and directly lateral to, the articular processes of the vertebrae so that the glenoid portion is directed ventrally. The body of the scapula extends along the side of the fetus over the three lower cervical vertebrae nearly to the angle of the third rib (fig. 1). This high or cephalic position of the shoulder girdle accounts for the horizontal course of the nerves of the brachial plexus. The shape and propor-

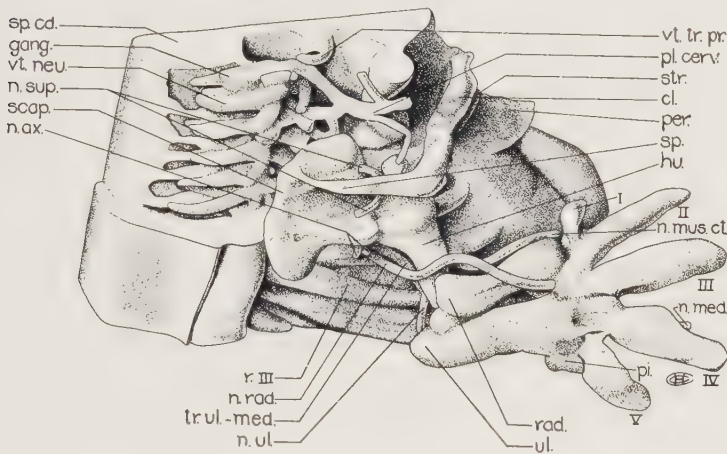


Fig. 1 Skeleton of fore-limb region, showing relation of nerve trunks. Drawn from reconstruction of fifteen-day albino-rat fetus.

tions of the scapula are very similar to that of the adult. The spine is well developed, separating the deep supra- and infra-spinous fossae, and the long acromion (figs. 1 and 4) joins with the clavicle as a continuous bar of precartilage. There is no apparent thinning to show the location of the acromioclavicular joint. The coracoid is much less prominent than the spine and the acromion. The head is not definitely marked off from the humerus.

The clavicle extends from the sternal anlage to meet the acromion and is well developed as compared with the other cartilages. From the midline to the acromion its length in

the reconstructed model is 62 mm., or longer than the humerus, and its average diameter is 11 mm., which is about the same as the radius. It consists largely of condensed tissue, but there is indication of ossification which resembles to some extent the process which takes place in membrane. Cartilage or precartilage is present also, particularly toward the medial end. This peculiarity of the cartilage of the clavicle and its ossification has been referred to by Mall ('06) and more recently by Jordan and Kindred ('26). The clavicles nearly meet at their medial ends, the line of union being shown by a slight thinning of the condensed mesenchyme. The sternum is evidenced only as the manubrium, in the form of bilateral extensions of the lower (caudal) parts of the medial ends of the clavicles diverging from the front of the pericardium and fading out in the direction of the tip of the first rib (fig. 1). However, these may represent very short sternal bars such as are described by Hanson ('19 a). These sternal anlagen extend scarcely as far along the thorax as the ventral ends of the first pair of ribs. Unless these may be so considered, there are, at this age, no sternal bands preceding the appearance of the presternum such as Hanson ('19 b) has described for the pig, especially, and for the mouse and other forms. The ribs end at the side of the thorax, so that the heart is covered ventrally only by the pericardium and integument (figs. 1 and 4).

The humerus (fig. 1) is very short and thick, the length in the reconstruction being 57 mm. and the average diameter of the shaft, 17 mm. The shaft is approximately parallel to the long axis of the body. The epicondyles and the fossae of the distal end are present. The head is not well marked off from the neck, but the greater tubercle is prominent. The forearm is almost fully pronated, so that the radius crosses the ulna (fig. 1). The latter is the more massive of the two. The respective lengths of the reconstructed ulna and radius are 60 mm. and 52 mm., while the average diameters of their shafts are 12 mm. and 10 mm. These, with the humerus, are very much shorter for their thickness than might be expected.

The head and neck of the radius are not clearly demarcated, but the olecranon and the coronoid of the ulna are prominent. At the wrist both join the carpal elements without clearly defined limitation.

The carpus shows irregular condensations of the mesenchyme, having very indefinite borders and with intervening thinner areas. Definite outlines of the separate parts are not to be distinguished. However, the pisiform does stand out as a projection. The digits (fig. 1, *I* to *V*) contain a core of young precartilaginous or dense mesenchyme, but here, too, the separate metacarpals and phalanges cannot be certainly described. Externally, the fingers show only as slight ridges between shallow grooves. This part of the skeleton is still merely the hand plate, as described by Lewis ('02).

There are no joint cavities. The precartilaginous gives way to condensed mesenchyme of the perichondrium at the ends of adjacent skeletal elements of the limbs, and in some cases there is a thinning of this mesenchyme at the location of the future joints. The mesenchyme around the thinner areas shows no indication of differentiation into capsular ligaments. Further observations are to be made to determine the age when definite joint cavities appear.

THE MUSCLES

The muscles of the shoulder are found to be well differentiated as to general form. Nearly all the muscles exhibit their usual attachment to the skeletal elements. The attachment in most cases is by means of massed mesenchymal cells elongated in the direction of the future tendons where they join the skeleton. These attachments are more definite and the differentiation of the muscle masses is more marked in the proximal parts. The description of the individual muscles is detailed below.

Muscles of the shoulder girdle

The m. sternocleidomastoideus (figs. 4, 5) shows two rather widely separated heads of origin, one from the medial end

of the clavicle and the sternal anlage, the other from near the middle of the clavicle. The two parts converge rapidly and pass to their attachment to the skull. The *m. omohyoideus* (figs. 4, 5) crosses the deep surface of the sternomastoid from the common infrahyoid mass to the scapular notch, where the suprascapular nerve passes in front of it. The common premuscle mass of *mm. sternohyoideus et sternothyroideus* passes behind the clavicle with little or no attachment thereto and terminates on the ventral end of the first rib. The *m. trapezius* has its usual attachment to the scapular spine and extends as a very thin layer over the back from the occiput as far as the seventh rib. No clavicular attachment is evident. The portion attached to the acromion, however, diverges from the remainder, and, passing upward behind the *m. sternomastoideus* and the jugular vein, gains attachment to the side of the first vertebra. To this slip in the wood rat Howell ('26) gives the name of *atlantoscapularis* (figs. 3, 4). In its course it separates the ascending branches of the cervical plexus from the remainder of the superficial branches of the plexus. The *mm. rhomboidei* (figs. 3, 4) are represented by a thin layer attached to the cephalic part of the base of the scapular spine and to the vertebral border cephalad to this. This muscle extends dorsally to become indistinguishable from the *m. trapezius*, with the exception of a rather wide slip of the cephalic part which passes anteriorly and more deeply and is attached to the cranium just medial to the mastoid. This portion is the *m. occipitoscapularis*. The *m. levator scapulae* (fig. 4) takes origin from the transverse processes of the cervical vertebrae as high as the third. It is continuous with, and indistinguishable from, the *m. serratus anterior* (figs. 2, 3, 4) below, and they insert without a break along the vertebral border of the scapula. The digitations of the *m. serratus anterior* are evident along the ribs between the *mm. pectoralis major et latissimus dorsi*. These digitations can be followed as far as the seventh rib. The *m. subclavius* (fig. 4) is a small slip which extends almost directly cephalad from the first rib near its anterior (ventral)

end to the caudal surface of the clavicle near its lateral extremity. The *m. pectoralis minor* (fig. 4) is a narrow slip inserted upon the lower part of the coracoid and is indistinguishable from the *m. pectoralis major* not far from their insertions.

Muscles acting on the shoulder joint

The *m. pectoralis major* (figs. 4, 5) has attachment for only a short distance along the medial end of the clavicle, but

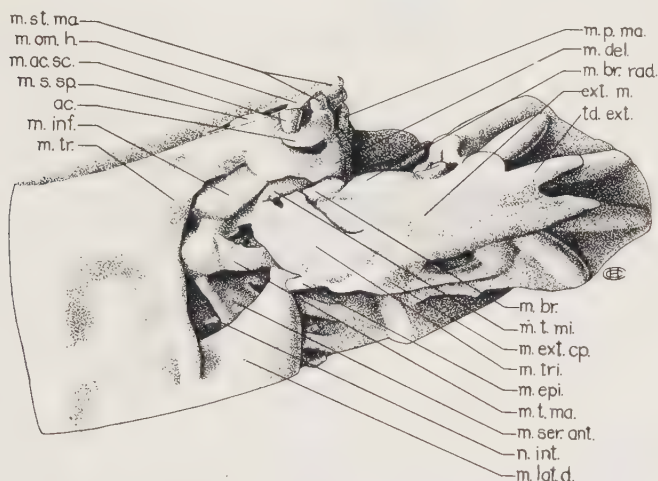


Fig. 2 Superficial dissection of fore limb, lateral view.

is widely spread over the side of the thorax as far as the fifth rib. At its lower border it overlaps the *m. rectus abdominis*. The *m. deltoideus* (fig. 2) has three separate slips from the scapular spine, the acromion, and the lateral half of the clavicle, respectively, and is inserted at the medial side of the *m. brachialis*. The *m. supraspinatus* (figs. 3, 4) is a bulging mass in the wide supraspinous fossa of the scapula, where it is covered by the *m. trapezius*. It passes beneath the acromion to be attached to the humerus, where it is covered by the *m. deltoideus*. The *mm. infraspinatus et teres minor* (figs. 2, 3, 4) appear in the infraspinous fossa

between the *m. deltoideus* and the *m. trapezius* and are demarcated from each other by a slight groove. They are inserted at the upper end of the humerus under cover of the *m. deltoideus*. The *m. teres major* (figs. 2, 3, 4) is rather widely separated from the *m. teres minor* and passes medial

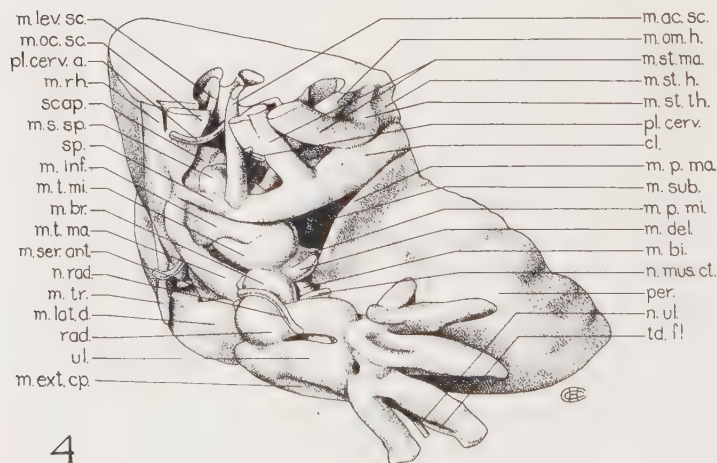
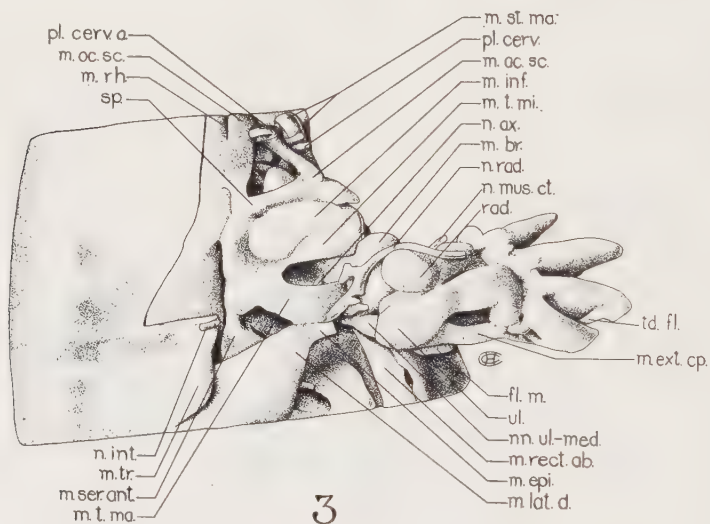


Fig. 3 Deep dissection of region of fore limb, lateral view.

Fig. 4 Deep dissection of fore-limb region, seen from above and in front.

to the humerus under cover of the m. triceps to be inserted on the medial side of the humerus along with the m. latissimus dorsi, which joins the m. teres major just before they are covered by the m. triceps. Near their meeting-point, a narrow slip of muscle passes from the m. latissimus dorsi to be inserted on the medial epicondyle of the humerus in close association with a portion of the m. triceps. Howell ('26) names this muscle the m. epitrochlearis (figs. 2, 3). Constant in the rat, it corresponds to the occasional m. epitrochleo-anconeus of man.

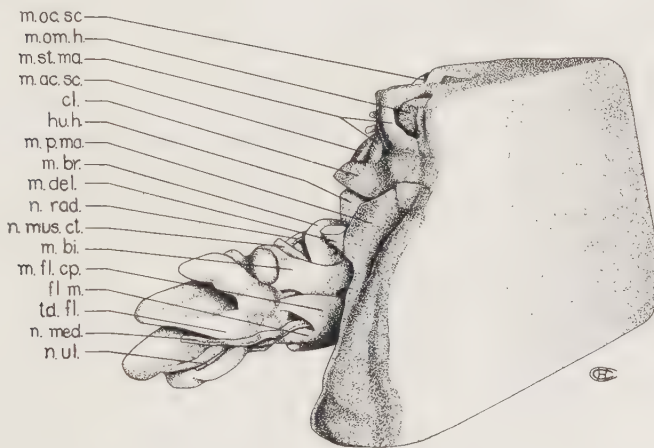


Fig. 5 Anteromedial view of deep dissection of fore limb.

Muscles acting on the elbow joint

The m. biceps (fig. 5) passes from deep to the insertion of the m. pectoralis major to be attached to the anterior surface of the radius. A portion which joins the anterior surface of the humerus near the middle of the shaft is the m. coracobrachialis. The m. brachialis is a bulging mass lying lateral to the m. deltoideus. It passes deeply under cover of the m. biceps to be attached to the proximal part of the ulna. The m. triceps (fig. 2) is a broad sheet overlying the dorsal and posterior parts of the arm and gaining attachment to the olecranon of the ulna, where it blends indistinguishably with

the common extensor mass of the forearm. The scapular head is well developed, passing between the mm. teres major et minor. The lateral and medial heads have the usual relationship on each side of the n. radialis.

Muscles acting on the forearm and hand

The flexor mass (fig. 5) is not differentiated into separate muscles at their origin, but deeper and more superficial parts of the tendons can be distinguished, especially in the palm, indicating mm. flexor digitorum profundis et flexor digitorum sublimis. The m. flexor carpi ulnaris (fig. 5) is fairly well defined, extending from the medial epicondyle to the attachment at the wrist.

The extensors (fig. 2), also, are represented by a common mass on the dorsum of the forearm, but the m. brachioradialis exhibits a separate tendon. The tendons of the m. extensor digitorum communis can be distinguished extending approximately halfway out on the digits, where they become indistinguishable from the mesenchyme.

The deeper muscles of the forearm and the intrinsic musculature of the hand are indefinite masses inseparable from the overlying muscles.

THE NERVES

The nerves, in relation to the skeleton and muscles, are seen to have practically the same origin from the brachial plexus as in man and the plexus is formed in essentially the same way. The plexus is derived almost exclusively from the lower four cervical nerves and the most cephalic of the four gives a larger component to the cervical plexus than to the brachial plexus, so that the latter is formed chiefly by the three lower cervical nerves (fig. 6). There is a contribution from the first thoracic nerve which crosses the medial surface of the neck of the first rib to join the eighth cervical nerve. This differs slightly from the condition found by Goering ('28), who worked on adult rats. Goering pictures the first thoracic nerve as the largest of those entering the plexus.

This difference may be within the normal limits of individual variation.

Due to the forward position of the fore limb, the nerves pass in relatively horizontal direction laterally and forward to form the trunks. The upper two and the lower two nerves, respectively, unite to form upper and lower trunks, which then join in a wide band. This almost immediately divides into an upper and a lower part, rather than into three cords.

From the upper part of the plexus are given off the *n. suprascapularis* and the *nn. subscapulares*. Before the division of the plexus, the *n. thoracalis longus* arises from the back

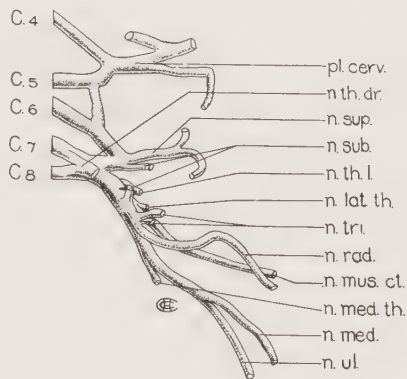


Fig. 6 Detail of brachial plexus, drawn from the reconstructed model. The component from the first thoracic nerve is not shown.

of the lower cervical nerves. The *n. dorsalis scapulae* arises from the most cephalic of the cervical nerves of the brachial plexus. It pierces the *m. levator scapulae*, giving off branches in its substance, and passes to the *mm. rhomboidei*. The *n. suprascapularis* (figs. 1, 6) takes its typical course anterior to the *m. omohyoideus* to encircle the neck of the scapula and pass into the fleshy parts of the *mm. supraspinatus et infraspinatus*. The *nn. subscapulares* pass directly into the *m. subscapularis* and *m. teres major*. The *n. thoracalis longus* passes downward upon the *m. serratus anterior* and branches into its substance. The lower lateral part of the plexus gives off all the remaining nerves of the fore limb.

Posteriorly from this part of the plexus arise the n. radialis and the n. axillaris. The n. axillaris (figs. 1, 4) appears between the m. teres major and the m. teres minor and passes into the substance of the m. deltoideus. The n. radialis (figs. 3, 4) gives off branches to the m. triceps and, under cover of this muscle, passes spirally around the humerus to come between the m. brachialis and the m. brachioradialis and into the forearm under cover of the extensor muscles.

Anteromedially, the n. musculocutaneus (figs. 4, 5) arises, pierces the m. coracobrachialis, and appears at the lateral border of the m. biceps to pass down the forearm superficially. Branches from it pass into the muscles named and into the m. brachialis. Cutaneous branches are given off in the forearm. Near its origin there is a small nerve which passes into the m. pectoralis major—probably the n. thoracalis anterior lateralis (fig. 6).

Between the anterior and posterior trunks as described, there is a larger middle portion (fig. 6) which gives a large branch to the mm. pectorales and then passes down the medial side of the humerus where, somewhat below the middle of the arm, it divides into two nearly equal branches, the n. medianus and the n. ulnaris. From the division the n. ulnaris turns posteriorly and, passing between the two heads of the ulnar flexor of the wrist (fig. 5), proceeds down the forearm and into the palm, giving off branches into the muscles and to the integument (figs. 4, 5). The n. medianus continues down the medial side of the arm to the elbow, where it passes deep to the muscles of the flexor mass, but reappears about the middle of the forearm and continues with the tendons into the palm (fig. 5). Muscular branches arise from it. The terminal branches of both the n. medianus and the n. ulnaris can be followed well out into the digits, where the terminal branches are given to the integument.

It is seen that the nerve trunks are remarkably developed, both as to size and extent, and that they have their usual relationships to the skeleton and muscles. As already stated, both muscular and cutaneous terminations are in evidence, even into the hand.

DISCUSSION

It may be noted that the degree of development, as here described for the rat, is attained much later, relatively, in the period of gestation than in man as described by Bardeen and Lewis ('02). Lewis shows that a human embryo having the skeletal, muscular, and nervous development corresponding to that which has been described above for the albino rat would be of about five weeks' development, or but one-eighth of the entire period of intra-uterine existence. It has also been noted by Strong ('25) that ossification in the rat begins much later, relatively, than in man. What significance these facts may have is problematical, but they agree with the fact that human spontaneous movements, known as 'quickening,' are recognized by the mother or by the physician before or near the middle of pregnancy and, sometimes, according to Williams ('23, '28), as early as the end of the first quarter. There may be a correlation between these two sets of facts. Moreover, Minkowski ('23), in admirable observations upon a series of human fetuses removed at cesarean operation, saw spontaneous athetoid or vermicular movements in limbs and trunk of fetuses toward the end of the second month. No muscular response, even after electrical stimulation, was seen in younger individuals (Minkowski, '28). How much earlier spontaneous movements are present in the human fetus or at what age muscular response may be elicited by stimulation will, probably, not soon be determined directly, due to the obvious difficulty of direct observation of these phenomena in man. But by comparison of equivalent physiological stages of development of human embryos of a given age with a series of fetuses of the albino rat or similar standardized research mammals, tested according to the methods of Swenson, a reasonable estimate of the time might be made. Accordingly, this investigation, which has not yet taken into account the finer structure, tentatively indicates the age of earliest motility of the human fetus to be in the sixth week.

It is pertinent, also, to consider here the relation of tissue differentiation to the entire absence of motility. Mall ('98)

and others have called attention to the fact that nerve and muscle are associated with each other from their earliest stages. Mall ('10) further remarks that nerves are indicators of the morphological origin of muscles, but that the nervous system does not influence muscle differentiation. Bardeen (Bardeen and Lewis, '02) states that the muscle differentiation immediately follows entrance of a motor nerve into a region, but he emphasizes that a causal connection must not be assumed to exist. Carey ('21 a, b, c) holds that all musculature is formed from the mesenchyme along lines of 'adequate intermittent tensile stress' which are elicited by an 'extrinsic growth force,' and by elaborate experiment ('24) shows that one variety of muscle can change to another in agreement with his theory. The present research shows that the muscles of this mammal, as well as the other tissues of the fore limb, progress normally toward their final form without any motility of the individual as a factor, and in so far is additional evidence of self-differentiation of the muscles which has been so well shown by the brilliant experiments of Harrison ('04) upon frogs and their more recent confirmation by Matthews and Detwiler ('26). In his researches upon the outgrowth of axones, Harrison ('10) further demonstrated that, even when isolated in a passive isotropic medium, "Each type of cell follows the same course of differentiation which it would have taken had it not been removed from the embryo, as is seen, for instance, in the formation of striated fibrillae in cells from the axial mesoderm," etc. Lane ('17), too, has called attention to the specificity of tissues in his work upon the special-sense organs of the rat.

SUMMARY AND CONCLUSIONS

From the detailed observations already made, it is seen that the skeletal parts are well developed as to form, rigidity, and relative position. The joints have not completed their development, there being neither joint cavities nor articular ligaments, but the continuation of the perichondrium between adjoining segments and the intervening rarefaction of the

mesenchyme separate the skeletal segments from each other. From these considerations it is reasonable to conclude that the skeletal parts are far enough advanced to afford attachment for muscles and to serve as levers in producing movement, and that movement is possible to some extent at the sites of mesenchymal rarefaction.

The muscles of the greater part of the limb are differentiated from the premuscle mass and exhibit their typical attachments to the skeleton, so that their contraction would bring about the typical movements. Their degree of fibrillation and striation and whether they have the ability to contract remains a problem for further investigation which is now in progress.

The nerve trunks arise in typical fashion from the brachial plexus and follow their usual courses even as far as the digits, giving off branches to the muscles contiguous to them. The branches break up among the bundles of fibers of the muscles, and the conduction path from the central nervous system is apparently complete. Whether the nerve endings have the necessary connections with the muscle fibers or whether neurofibrillae are yet formed, as Lane ('17) believes to be the deciding factor with regard to the special-sense organs, remains to be determined.

It is seen that the gross structural parts of the shoulder, arm, and portions of the forearm are prepared for movements of the fetus. Yet there are no movements of any kind up to this age. It would seem that we have here the static assembling of many bodily components which await some complementary addition to render them a dynamic whole. And the advanced degree of development that has been shown by this investigation to be attained before movements occur helps to explain the sudden appearance and subsequent rapid spread of motility. Further light on the problem of fetal behavior will be sought, as already indicated, by continuing the investigation with the specialized histological methods and by means of direct experiment upon the muscles concerned.

Grateful thanks are extended to The Wistar Institute and its Director, Dr. M. J. Greenman, who have provided facilities for pursuing this work. Also a deep debt of gratitude is acknowledged to Dr. George E. Coghill, whose advice and constructive criticism have made the completion of this portion of the undertaking possible.

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EXPERIMENTS IN SPLITTING THE REGENERATING LIMB BUD¹

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FIVE FIGURES

INTRODUCTION

The production of double limbs has often followed various types of injury to the regenerating bud in a number of different animal groups. In the Crustacea and the Amphibia this phenomenon has been particularly noticeable and has followed operative, as well as accidental, injuries. Without detailing the older experiments at this time, it will suffice to call attention to the general results obtained. In most cases the members of a reduplicated limb complex produced by injury were mirror images of each other, but in others they were not. Nearly, if not quite, all of the instances observed in nature conformed to the rules set forth by Bateson ('94), but a few, especially among the experimentally produced examples, consisted of limbs in series (Swett, '24 b). In some instances no clear statement of the exact origin of the regenerating blastemas or of the asymmetry of the resulting limbs has been given. In an attempt to check over the possibilities and reconcile certain discrepancies, the present study was undertaken.

Although no solution of this problem has yet been reached, it has seemed advisable to publish such results as have been obtained, in the hope that the facts presented may be of

¹ This work was carried out at the Osborn Zoölogical Laboratory, Yale University, in 1920. The problem was undertaken at the suggestion of Prof. Ross G. Harrison.

some value in relation to the recent work of other authors. I refer especially to the observations of Graper and of Weiss, who studied the regeneration of the limbs after longitudinal bisection of the limb buds. Graper ('26 a) amputated the manus and pes of many young axolotls and split the zeugopodium lengthways, or in some cases divided the regenerating bud with a thread. Normal or nearly normal extremities resulted, the variations consisting of split or supernumerary toes. In ten animals a celluloid cap was sewed in place over the tibial or fibular portion after the stump had been split. This served to keep the halves apart longer and prevented, in some degree, the fusion which characterized the first experiments. He has described nine and figured seven of the cases. After regeneration, two cases showed five toes, one case six, three cases seven, two cases nine, and one case eleven. His statement that one of them, a lateral (seitlich) regenerate, was formed by proximal regeneration implies that it was the mirror image of the main member. Otherwise, no mention was made of the asymmetry of the supernumerary parts produced.

Graper ('26 b) also found that extra limb parts were produced when small (1, 2/1) limb buds of *Bufo* were split into tibial and fibular parts (dorsoventral split) and one portion left in place while the other was transplanted into the tissue of the tail. Results in this type of experiment were somewhat variable, owing to the tendency of the bud parts to be resorbed or to produce deficient or otherwise abnormal limbs. His best case, from the present point of view, is discussed at some length. In this instance the portion of the bud grafted to the tail formed a fairly well developed limb with a reduced number of toes. From the other part a well-developed leg with a hyperdactylous proximal regenerate was produced. This case resembles those described by Tornier ('05).

Weiss ('26) has presented a series of experiments on *Triton cristatus* in which the limb was split, one or both portions then being amputated and fusion of the parts prevented. He found it somewhat difficult to get growth from

each surface, even though his technique insured their fairly wide separation. He amputated the limbs at the wrist (ankle), split the zeugopodium, then, 1) cut off the postaxial half at the elbow (knee), forming a proximal cut surface ('p-Fläche'), while the sides of the preaxial half were covered by the skin removed from the other. Its tip formed the distal cut surface ('d-Fläche'). Or, 2) the stump of the postaxial half was left as the distal cut surface, the preaxial being amputated at the middle joint and presenting there a proximal cut surface. A further series, 3) consisted of a simple split of the zeugopodium, both halves remaining.

In these experiments, a complete regenerate was produced in some instances from one of the two half cross-section surfaces, the other not growing at all. The cross-section topography of such a complete regenerate from a half stump seemed to bear no part for part relationship with the constitution of the cut surface.² In a considerable number of cases regenerates developed from both cut surfaces. Three classes of these were noted. A. A complete regenerate was sometimes developed from one surface; from the other, a small digitless tip. B. If individual development at each surface began and fusion then took place, the limb so produced was said to be made up of a complete regenerate from one surface and a partial regenerate from the other. C. Of partial regenerates from both surfaces there were two kinds. The first was the result of the early fusion of two blastemas to form one complete regenerate. In the second, the two components remained separate and each bore at its tip a half regenerate. These limbs seemed to grow as readily from one portion of the stump as from the other.

In none of this work was the asymmetry of either of two regenerates from neighboring distally directed cut surfaces reversed. The only reversed limbs were classed as proximal regenerates. Seemingly, this does not harmonize with some

² A similar condition has been reported in *Amblystoma* (Swett, '24 a). The tendency of regeneration, after amputation of irregular and massive reduplicated complexes, was in the direction of normal single limbs.

of the older work, notably that of Tornier ('05), who reported reversal of asymmetry in one of the limbs resulting from a split bud in *Bombinator*, of Przibram ('02), Reed ('04), and Zeleny ('05), who got mirror-imaged limbs from a split rudiment in the Crustacea, of Megusar ('07) on the Coleoptera, and of Kurz ('12, '22), experimenting on Triton. The results agree, however, with another group of experiments in which two limbs in series resulted from a split rudiment (Swett, '26). This work has recently been presented, and a brief review here will suffice. The limb rudiment of *Amblystoma* embryos in the tail-bud stage was divided in one direction or another into approximately equal halves. These halves were prevented from fusing by the transplantation of a strip of indifferent tissue into the wound. In every case the primary limb from each half was harmonic, two limbs in series being produced in a certain number of instances. The only limbs of reversed asymmetry were those supernumerary members which were produced, under certain conditions, by one of the primary limbs. Varying the plane in which the rudiment was divided seemed to make no difference in the result. The irregular formations produced when older buds were divided resembled more closely those derived from attempts to split regenerating blastemas.

The experiments reported here serve merely to augment the cases in which division of the regenerating blastema has been practiced and to point out certain factors which must be controlled in future work. The inconsistencies must remain, for the present, without explanation.

MATERIAL AND METHODS

Adult *Diemyctylus viridescens* (Raf.) were used because of the ease with which they could be secured and the readiness with which they lend themselves to operative procedure. They are easily handled and the limbs regenerate at a fair rate of speed. The animals were fed on earthworms, frog tadpoles, or flies and they were found to live for a considerable length of time and regenerate at a nearly normal rate without food of any kind.

In these experiments the limbs were amputated fairly close to the side of the body and the animals placed in a dish lined with moist filter-paper until the wound healed. Amputation was accomplished by simply snipping off the limb with scissors. In some cases slight hemorrhage followed the operation, but this was never great enough to be serious, and in most cases the scissors pinched the edges of the wound together firmly enough so that very little bleeding took place. At varying intervals after the wound had healed, the regenerating surface was divided by one method or another. This second operation was done under chloretone (1-1000 solution) anesthesia. At first it was attempted to fashion a harness of silk thread which should pass around the body of the animal, with one loop dividing the wound surface at the desired angle. This was unsuccessful, because in those few cases in which the animal did not succeed in slipping out of the harness, the binding threads caused a marked edema and had to be loosened. It was found better to sew the thread into the skin above and below the regenerating area with a strand passing over the surface to be divided. In some cases a small notch was cut in the tip of the regenerating bud, so that the thread would not slip out of place. In other cases the regenerating bud was slit in the desired direction with fine-pointed scissors, and a fragment of bone from the stump or a piece of muscle from the tail of the animal was inserted. In a few of the later experiments the tail was amputated at the same time as the limb and a portion of the regenerating tail blastema transplanted to the regenerating limb bud to keep the two portions apart. Although only a few operations of this kind were done, this technique promised to have some advantages over the others.

EXPERIMENTAL

A total of 110 operations were performed, using one or another of the methods outlined above. A normal limb was regenerated in all cases except seven. In four of the seven cases which showed some degree of doubling the limb was

hyperdactylous, and in three cases a reduplicant was formed to which a definite asymmetry could be assigned. Although the number of successful experiments was small, a brief description of each will be given here simply for the purpose of recording the anatomical relations of the parts.

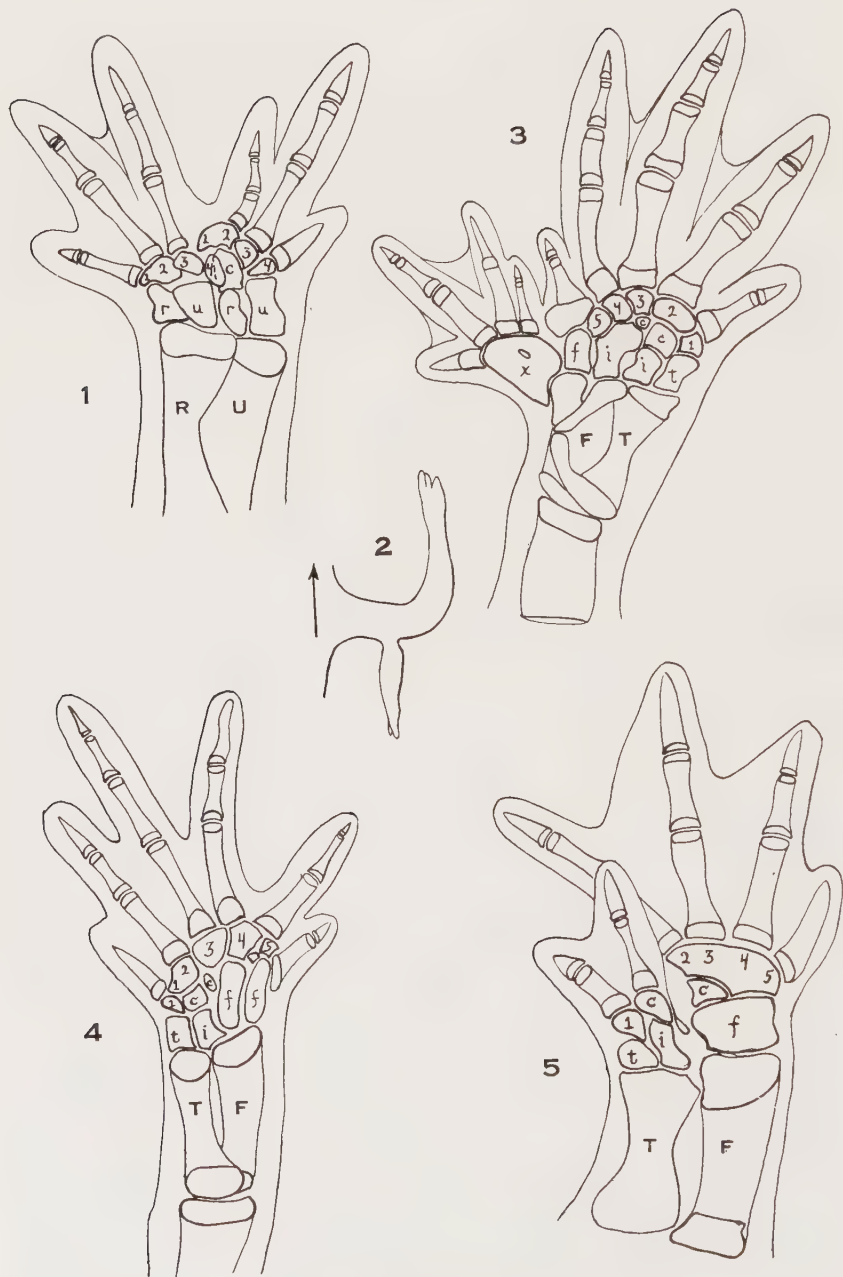
In the first case to be described a series of operations were performed on the right fore limb in an attempt to split the regenerating bud. The result was the formation of the complex shown in figure 1. Two limbs were present. These were mirrored in the dorsal plane, the more posterior being a right and the anterior a left. The first digit of the posterior member and the fourth digit of the anterior member are not visible in the figure. Both of these appeared in the angle between the two limbs during the course of development, but gradually became resorbed. The arrangement of the carpalia in the diagram would give the impression that the two limbs were in series, but, as a matter of fact, the cartilages shown on the observer's left were situated in a plane elevated somewhat above those on the right. The doubling was confined to the parts distal to the forearm and involved all those parts except the centrale. This cartilage was single and common to the two limbs. The function of this complex was perfect except that the opposed dorsal surfaces were used as palmar in locomotion. No doubt this was due to the lack of flexibility in the wrist caused by the duplications in this region.

In another case the bud regenerating on the stump of the right fore limb was divided dorsoventrally by means of a strand of silk thread, three days after the amputation. The two limbs which developed are shown in figure 2. This animal died and was lost before an anatomical study could be made, but observations on the living animal showed the main limb was a normal right and that the smaller one, which came off the other near the point where the amputation was made, was a small, abortive left. The elbow showed plainly on both, but distal to this the smaller limb was flaccid, indicating a lack of chondrification. This component was not observed to function. The drawing is from a sketch made from life thirty-eight days after the operation.

In a third experiment the stump of a right hind limb was divided dorsoventrally five days after amputation and a fragment of tissue from the tail inserted into the wound. One month after this procedure a fan-shaped structure was noted on the dorsal surface of the regenerating limb. This developed into a small limb which was a fibulo-dorsal mirror image of the larger functional regenerate. Figure 3 shows the relationship between these limbs and the arrangement of the cartilage of the tarsus. The doubling involved the tarsus particularly, with evidence of some compounding in the shank. The duplication of the centrale and intermediale of the larger limb was not necessarily a consequence of the operation, as there is normally considerable variation in these elements.³ The supernumerary pes in this complex articulated with a special part of the distal fibular epiphysis and presented a cartilaginous structure composed of fused tarsal elements whose component parts could not be distinguished. The function of the appendage as a whole was very nearly normal, although there was evidence of stiffness at the knee-joint. The smaller member had no function independent of the larger.

Two of the cases of hyperdactyly presented only a very minor duplication in one digit, but the other two exhibited sufficient involvement of the cartilages of the tarsus to make them worthy of mention. In both of these cases the right hind limb was the one affected, and the injury resulting in the condition described below followed the constriction of the regenerating bud by means of a silk thread. In the first case (fig. 4) the fifth digit was doubled. Aside from this, a double fibulare was the only deviation from the normal, the other slight changes from the typical arrangement coming within the limits of normal variation. In the other case (fig. 5) rather marked changes were produced in the tibial side of the limb. The duplication involved the first digit

³ Normally, the tarsus consists of a proximal row of cartilages which includes the tibiale, intermediale, and fibulare, a variable (single or double) centrale, and a distal row of five tarsalia.



Figures 1 to 5

which articulated with the tarsal cartilages belonging to this side of the limb. Articulating with the fibula was a single large cartilage, composed of a fibulare which may have been compounded with part of the intermediale. Distal to this was a large bar of cartilage, composed of the fused tarsalia nos. 2 to 5, which articulated proximally with the fibulare and centrale and distally with the corresponding basal phalanges. There was a considerable separation between the two tibial digits and the rest of the limb. The former in life assumed a somewhat acute dorsal projection.

DISCUSSION

The limbs developed in connection with the present series showed a duplication of parts which was confined chiefly to their distal extremities. The tip of the conical bud which developed from the wound surface seemed destined to produce the distal end of the free appendage, further growth in length taking place mostly from the base of the blastema. This necessitates an early division of the bud if a complete separation is to be secured. Independence of the two limbs was more nearly approximated by the experiments of Weiss ('26) and Graper ('26 a) when they permitted growth from two surfaces at different levels or definitely separated by other means. In their cases, as in the ones reported here, fusion of the growth centers took place if they came in contact with each other at almost any stage in the regenerative process. This tendency recalled the results of similar experiments (Swett, '26) on the embryonic limb rudiment. It was found impossible to secure a limb from each half of such a

Camera-lucida drawings made after the parts were stained with methylene blue and cleared in cedar oil. Magnified. Descriptions given in text. *F*, fibula; *T*, tibia; *R*, radius; *U*, ulna; *f*, fibulare; *t*, tibiale; *r*, radiale; *u*, ulnare; *i*, intermediale; *c*, centrale; *x*, fused tarsal elements. 1, 2, 3, etc., first, second, third, etc., tarsalia (carpalia).

Fig. 1 Dorsal view right fore leg, experiment RD3.

Fig. 2 Dorsal view right fore leg, experiment RD-Ser X.

Fig. 3 Plantar view right hind leg, experiment RD-Ser Z.

Fig. 4 Dorsal view right hind leg, experiment RD5.

Fig. 5 Dorsal view right hind leg, experiment RD-Ser D.

divided rudiment unless a definite barrier of indifferent tissue was interposed to prevent fusion of the separated parts and the consequent production of a single, normal limb.

One of the cases reported here (fig. 5) resembles those described by Weiss in which a partial regenerate was derived from each surface, the portions remaining more or less separate distally throughout development. The case shown in figure 3 might be classed with Weiss' group in which a complete regenerate was derived from one surface and a partial regenerate from the other. Possibly, also, the group containing the hyperdactylous limbs might be classed likewise. The limbs shown in figures 1 and 2, however, although seemingly only partial limbs in their final form, should probably be placed in a group which was not presented in Weiss' experiments. In each of these cases two complete limbs were developed. They were quite definitely mirror-imaged and thus fell into the same category as those in many experiments previously described.

However, it was impossible to determine, because of the nature of the operation, the exact derivation of the two growth centers in these cases. The methods used to divide the bud were so unsatisfactory that the operation really constituted more of a general disturbance to the regenerating bud than a simple bisection. The techniques used by Weiss and by Graper in the experiments referred to above, gave a much better chance to control the bud origins, because of the wider separation of the growing parts. Direct distal regenerates, in their experiments, apparently gave no evidence of reversal of asymmetry. In most of the cases one of the buds developed into a partial limb which varied from one to several digits. The studies on the embryonic limb have shown that simple division of the rudiment, with permanent separation of the parts, did not result directly in asymmetry reversal. Both of the primary limbs derived in this manner, one from each portion of a bisected rudiment, belonged to the same side of the body. Members of opposite asymmetry were secured in these experiments, however, by spontaneous

reduplication of the primary members. Also, in the divided and then disoriented rudiments (Swett, '28), the unusual factors of recovery of posture by rotation, of abnormal growth and of regeneration, played a definite rôle in producing limbs which were mirror images of their respective primary members.

From the results of all the experiments so far reported, there seems to be some doubt whether a true pair of limbs, i.e., a right and a left, have ever arisen as primary members from separated, but not disoriented portions of the same blastema. The changes, obviously initiated by certain types of operations upon, or injuries to, the embryonic or regenerating bud, and resulting in the production of mirror-imaged members, are still not understood. Graper ('26 b) has postulated that certain bonds ('Bindungen') within the limb are interrupted by operations such as those which divide the rudiment, and that reverse growth along the severed lines results in the formation of the reversed member as a proximal regenerate. If such a mechanism exists, one must infer that all operations which result in the production of mirror-imaged limbs somehow interrupt it, while those which produce two limbs of the same asymmetry must either have failed to injure it or, in these instances, proximal regeneration must have been inhibited. That the stage of development or the degree of differentiation of the rudiment does not materially influence the result is evidenced by the number of experiments on the same stage which have given conflicting evidence.

One must, it seems, conclude that the factor which causes, or produces, reversal of asymmetry in one of two buds derived from a single anlage, is fundamentally independent of the mechanical and artificial bisection which seems only to produce two rudiments essentially alike so far as their asymmetry is concerned.

SUMMARY AND CONCLUSIONS

The experiments reported consist of five limbs doubled in different degree as a result of attempts to divide the regenerating limb blastema in *Diemyctylus viridescens*. In three of them the asymmetry of one member of the complex was reversed. No evidence was secured, however, which indicated that the artificial bisection was the direct cause of this reversal of asymmetry.

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THE HISTOLOGY OF THE THYMUS GLAND OF THE BOX-TURTLE, *TERRAPENE CAROLINA*, WITH SPECIAL REFERENCE TO THE CONCENTRIC CORPUSCLES OF HASSALL AND THE EOSINOPHILIC GRANULOCYTES

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ONE COLOR PLATE AND FOUR HELIOTYPE PLATES (THIRTY FIGURES)

INTRODUCTION

The recent studies of thymic corpuscles by Kingsbury ('28) and by Dearth ('28) have introduced a new conception regarding the significance of these peculiar structures. These investigators interpret the concentric corpuscles of Hassall of the mammalian thymus in terms of the expression of the growth of an epithelium with a tendency to keratinize in the absence of a free surface. Jordan and Horsley ('27) presented what they regarded as strong evidence in support of Cornil and Ranvier's ('73) original interpretation of these corpuscles as areas of segmental obliteration of blood vessels in an involuting organ. It cannot be denied that at least occasionally the concentric corpuscles and blood vessels are intimately related (His, '62; Afanassiew, '73; Watney, '82). Moreover, the presence of a network of reticulum fibrils in certain typical corpuscles demonstrates that these elements are not composed solely of epithelial constituents (Jordan, '27). Obviously, similar morphologic conditions would result, whether blood vessels, with their accompanying reticulum, grew into concentric corpuscles; or whether such corpuscles formed in or around blood vessels. In such instances the question narrows down to one of primary focus or factor;

that is, whether intravascular or extravascular cells take developmental precedence.

In an effort to advance the analysis of the question of the significance of thymic corpuscles we have undertaken an investigation of these elements in the thymus of the common box-turtle, *Terrapene (Cistudo) carolina*. Here conditions are relatively simple, in that the majority of corpuscles are of the unicellular type. If these corpuscles are in fact hypertrophied endothelial cells, it should be possible to find clear evidence in this material. In favor of their vascular origin are: the fact that the unicellular forms are closely enveloped by a nucleated membrane very similar to endothelium (figs. 1, 2, 28, and 29) and, like endothelium, intimately related to reticulum fibrils (compare figs. 13 and 14); the occurrence of an occasional corpuscle in the cortex of the thymus lobule; and the similarity between multicellular corpuscles of the cystic type (fig. 10) and blood vessels whose lumen is becoming obliterated through hypertrophy of the lining endothelial cells (fig. 11). Furthermore, there occurs a definite numerical reciprocal relation between medullary thymic corpuscles and blood vessels. The opposing evidence concerns the relatively enormous size of many of the unicellular corpuscles as compared with capillary lumina (figs. 1 and 2); the occurrence of stages in the dissolution centrally of certain multicellular plasmodial corpuscles (figs. 6 to 9); and certain appearances suggesting a healthy active condition of the unicellular corpuscles as indicated by features resembling Golgi nets and mitochondria (figs. 3, 4, and 5) and vague evidences of atypical mitoses (fig. 12).

It is the primary object of this study to discriminate between genuine thymic corpuscles and possible simulacra, and on the basis of the accruing data to formulate a satisfactory and comprehensive interpretation of the significance of the thymic corpuscles of vertebrates. This object assumes an essential developmental identity of thymus bodies among vertebrates, as compounded of entodermal epithelial reticulum, lymphocytes, and blood vessels with reticular connective

tissue. The studies of Shaner ('21) and of Johnson ('22) give sufficient warrant for this assumption, at least as concerns reptiles and mammals.

This material gives also unexpectedly clear-cut data regarding the genetic relationship between lymphocytes and granulocytes, between basophilic and eosinophilic granulocytes, and between lymphocytes and plasma cells and Russell-body cells (figs. 15 to 26). This evidence will be briefly outlined and discussed at the end of the paper.

MATERIAL AND METHODS

The material of this investigation consists mainly of the thymus of the common box-turtle, *Terrapene (Cistudo) carolina*. Several other testudinales were investigated, with essentially identical results. The tissues were fixed in the Zenker-formol solution of Helly; and the paraffin sections stained, some with hematoxylin and eosin, some with the eosin-azur mixture of Giemsa, and some with the Foot technique for reticulum. Some tissue was treated also according to the Kopsch osmic-acid technique for the demonstration of mitochondria and Golgi apparatus.

DESCRIPTION

Figure 1 illustrates a typical unicellular corpuscle of large size. It is closely enveloped by a layer of flattened cells resembling endothelium. This membrane includes reticulum fibrils (fig. 14), demonstrating its mesodermal origin. The outlying cells include lymphocytes, reticulum cells, and smaller (younger) epithelial (entodermal) cells. The last-named are characterized by a paler nucleus and a generally larger, very irregular cytoplasmic body. The cytoplasm of these epithelial cells may extend into long irregular processes, which may anastomose with similar processes of adjacent cells.

The nucleus of the unicellular corpuscle of Hassall is generally located near the center of the cell. It is a pale-staining irregular structure, generally without sharp delimitation be-

tween chromatin and nuclear sap, except for the remnant of a nucleolus (fig. 29). The finely granular character and irregular shape of the nucleus mark it as that of a degenerating cell. The cytoplasm is acidophilic, more or less hyaline in appearance. It is further characterized by the possession of closely spaced concentric striae (figs. 28, 29, and 30). This condition renders the designation 'concentric' thymic corpuscle very appropriate. Under very high magnification these striae appear to be lines of fracture. This interpretation is supported by the fact that some corpuscles appear broken up into a small central nucleated mass and enveloping crescentic and globular masses (center of fig. 2). The corpuscles ultimately disappear by a process of liquefaction and resorption. An occasional bit of parathyroid may occur to confuse the picture.

Figure 2 illustrates a small area with many encapsulated unicellular corpuscles. Such areas cannot be said to be common. The corpuscles generally occur more widely scattered. In the upper portion of the field occurs a bicellular corpuscle. The overlying stellate nucleus is that of an encapsulating reticulum cell. The stoutly bilobed character of the corpuscle, together with the appearance of a narrow cleft at the right, suggests a process of fusion. However, corpuscles like that of figure 12 show that bicellular and binuclear corpuscles may arise also by division.

The accompanying cells of this group of concentric corpuscles are lymphocytes, reticulum cells, a few eosinophilic granulocytes and epithelial stromal cells. The last-named are potential precursors of concentric corpuscles. The lymphocytes can be readily identified by their deeper-staining nucleus and the block-like distribution of their chromatin. Many of these cells have certain nuclear and cytoplasmic characteristics which are by certain investigators (e.g., Maximow, '27) regarded as diagnostic of young plasma cells (figs. 15 and 16). The smaller epithelial stromal cells cannot be clearly differentiated from the reticulum cells in ordinary preparations. That many of these stromal cells, however,

are true reticulum elements is shown in the sections prepared with a special reticulum technique (fig. 14). As the epithelial cells hypertrophy in the process of transformation into Hassall corpuscles, they encroach upon adjacent reticulum cells and force these into an encapsulating membrane (figs. 28 and 29). An early stage in this process is shown at a point slightly to the left of center in figure 2. There is no indication of blood-vessel involvement in this process.

In sections prepared with the Foot technique for reticulum many of the corpuscles have an appearance like that shown in figures 3 and 5. The cytoplasm appears latticed. The meshes of this lattice are strikingly regular. The bars of the lattice are apparently lines of more extensive precipitation of silver salts. The Foot technique for reticulum is theoretically an appropriate silver-impregnation method for the demonstration of Golgi apparatus. To test the surmise that the lattice of the corpuscle cytoplasm might be a Golgi net, tissue was prepared according to the Kopsch osmic-acid technique for mitochondria and Golgi bodies. The result is illustrated in figure 4. Here the lattice is not so clearly outlined. The picture differs from that of figure 5 in that only the radial bars are distinct. The concentric bars are largely lacking.

Efforts to homologize the appearances of the cytoplasm of corpuscles stained by the Giemsa method (fig. 1), Kopsch method (fig. 4), and the Foot method (fig. 5) lead to nothing very satisfactory. One method emphasizes a concentric striation, the other a radial, and the third emphasizes equally both radial and concentric lines. In view of the fact that Golgi nets, at least of the size and form shown in figures 4 and 5, would suggest a high degree of virility, it seems very improbable that these striations could have this significance in these corpuscles marked by other features clearly indicating degeneration. It must, therefore, be concluded that the cytoplasmic patterns outlined in the sections prepared by the Foot (fig. 5) and the Kopsch (fig. 4) techniques have no other significance than areas of more extensive staining of

the striations, largely concentric, shown in ordinary preparations (figs. 1 and 2). This further leads to the tentative conclusion that these striations, which stain blue after eosin-azur (figs. 28, 29, and 30), are more fluid areas, possibly fluid-filled clefts, of relatively acid reaction.

As already stated, the prevailing type of concentric corpuscle is the unicellular. This is generally scattered at random, a few even occurring in the cortical region of the thymic lobule. Only rarely does it occur in nests as in figure 2. There occur also occasional examples of plasmodial (figs. 6 and 7) and cystic (figs. 8, 9, and 10) corpuscles. A closely graded series of transition stages shows the origin of the plasmodial or multicellular types as fusion products of originally discrete epithelial stromal cells (fig. 6), and the genetic relationship between the follicular or cystic varieties and the plasmodial corpuscles. These multicellular forms (fig. 6) resemble closely those described by Popoff ('27) in his tissue cultures of rabbit's thymus. Neither is genetically related to blood vessels. Such corpuscles contain no reticulum fibrils.

Figure 7 shows an early stage in the transformation of a multicellular corpuscle into one of follicular character. The central cells suffer liquefaction and thus effect the formation of a central lumen. Frequently, the process of dissolution is initiated more peripherally, producing a lumen with a plasmodial corpuscular content (figs. 8, 9, and 10). Such a structure resembles closely sections of atretic blood vessels in which the endothelial cells have hypertrophied and partially fused (fig. 11). However, these structures (figs. 9 and 11) are only simulacra one of the other, and there appears no unequivocal evidence of genetic relationship. The same conclusion pertains even to cases in which the follicular lumen contains eosinophils (fig. 10), and the resemblance to a blood vessel is still closer. The presence of eosinophils in the lumen of the follicular corpuscles is the result of a secondary invasion. Corpuscles similar to that of figure 10, but somewhat larger and with a large content of mixed cells essentially identical with the general parenchyma, are to be interpreted

as small areas of the parenchyma enveloped by epithelial stroma.

Figure 30 illustrates a type of corpuscle which occurs in small numbers. It at once suggests a unicellular corpuscle within a capillary lumen. The corpuscle might be thought to represent a hypertrophied endothelial cell. However, since no evidence can be found of such mode of development, this type of corpuscle must be given a different interpretation. We interpret it in terms of figures 8 and 9. As in the case of multicellular corpuscles, so also in corpuscles of the unicellular variety, a subperipheral liquefaction of the cytoplasm may separate an exoplasmic capsule from a central nucleated endoplasmic area. A similar condition appears in the case of the central corpuscle in figure 2.

Quite a number of corpuscles occur somewhat similar to that of figure 12. This corpuscle contains two nuclei interconnected by a delicate chromatic thread. The end result may be predicted as a binucleated corpuscle. In certain cases the division more closely simulates mitosis, in that the poles of the figure contain more sharply outlined threads and rods, resembling chromosomes. The most plausible interpretation of these conditions would seem to be one in terms of an original abortive effort at mitotic division in pathologic hypertrophying epithelial cells, the later stages resembling more an amitotic figure. In the tissue cultures of thymus of rabbit, Popoff ('27) describes active mitosis in the epithelial stromal cells.

In order to test further the possible relationship between thymic corpuscles and blood vessels, sections were prepared by the Foot technique for reticulum. The results may be illustrated by figures 13 and 14. Figure 13 contains no corpuscles. It was selected because it includes several cross-cut smaller blood vessels. The wall of the small vessels is sharply outlined by a reticular membrane. The wall of the larger vessels has a double reticular membrane, one next the lumen, a second membrane on the periphery. The difference is clearly shown at the lower border of the figure, where a

vessel at the right, with a double reticular membrane, passes into a branch at the left where only a single reticular membrane appears about the lumen.

The reticulum along the upper border belongs to the capsule. The capsule is continuous with a coarse trabecula at the right. The reticular stroma is especially coarse and abundant in the trabecula. The trabeculae are further marked by an unusual abundance of eosinophils. It seems clear from this figure that, outside of capsule and trabecula, the reticulum is practically restricted to the walls of the blood vessels. Only occasional threads appear to extend into the non-vascular areas of the parenchyma. This being so, an area like that shown in figure 14 becomes significant. Here the general parenchyma is well supplied with reticular stroma. Turtle tissue is strikingly refractory to the Foot method of staining reticulum. It is just possible that the area of figure 14 is a more successful result than the one illustrated in the area of figure 13. The area of figure 14 shows two unicellular thymic corpuscles. Both are closely surrounded by a reticular membrane, as if enveloped by endothelium. Moreover, the corpuscle in the upper right-hand corner of the figure is closely related to the obliquely cut blood vessel. Taken by itself, this figure might be used to support an interpretation of these corpuscles as hypertrophied endothelial cells. But the countervailing evidence from other sections seems too strong to warrant entertaining the possibility of such interpretation. The pericorpuscular reticulum membranes of figure 14 should probably be interpreted as belonging to stromal (mesodermal) reticulum rather than to endothelium.

The thymus of the box-turtle is, furthermore, an exceptionally favorable material for the study of the question of relationships between lymphocytes and granulocytes, lymphocytes and plasma cells, and between basophilic and eosinophilic granulocytes. Especially in the trabeculae and the subcapsular regions occur more numerous certain lymphoid cells characterized by a relatively wide shell of basophilic

cytoplasm (figs. 15 and 16). These cells suggest the plasmoidocyte of Maximow. While it remains uncertain whether all of the granulocytes arise in situ or whether many of them have migrated to the thymus, it is perfectly clear that some at least develop from local lymphocytes. The initial granules are relatively small, of spheroidal form, and have a basophilic staining reaction (fig. 17). The granules ripen into acidophilic conditions.

At this stage, the granulocyte is a typical eosinophil with spheroidal granules (fig. 19). Intermediate stages show a mixture, in varying proportions, of basophilic and eosinophilic spheroidal granules (figs. 18 and 22). The eosinophilic granulocyte with spheroidal granules metamorphoses further into the predominating type with ellipsoidal and bacillary granules and a polar oval or polymorphous nucleus (figs. 21 and 22). The transitional stage is characterized by a mixture of spheroidal, crescentic, and bacillary eosinophilic granules (fig. 20). The bacillary granules arise from spheroidal granules by a process which appears to be chiefly a matter of partial collapse of a fluid globule, or perhaps a rearrangement of the less fluid portion of the globule to effect an elongation into a rod form. While the nuclei of the lymphoid ancestors of these granulocytes have somewhat the pattern of young plasma-cell nuclei, there is no good reason for regarding the definitive granulocyte stages as in any sense abnormal. The enormous aggregation of granulocytes in the trabeculae of this thymus would appear to have some special functional significance. What this may be, however, we have no data upon which to base a very plausible suggestion.

It seems quite within the range of expectation that, under conditions where granulocytopoiesis is very active, a portion of the process should be abortive. Some lymphocytes fail to become granulocytes and degenerate into typical plasma cells (figs. 23, 24, and 25) and ultimately into Russell-body cells (fig. 26). Another peculiar, possibly pathologic cell occasionally found is a polynucleated element with red-staining crystalloids. Figure 27 illustrates the typical condition

of this cell. It is generally enveloped by a thick layer of epithelial (entodermal) plasmodium, somewhat like the cell of figure 30. This type of cell is found so sparingly that we have been unable to gather sufficient data to hazard a definite conclusion. We incline to regard it as of lymphocyte derivation.

DISCUSSION

The chief object of this study of the box-turtle's thymus is to secure data to aid in the interpretation of the more complex types of concentric corpuscles of the mammalian thymus. In the turtle the unicellular variety of corpuscle greatly preponderates. In its development it bears no primary relation to blood vessels. It does not represent a hypertrophied endothelial cell of a capillary in a region of involution. Secondly, as it increases in size, it may press upon and occlude a blood vessel. Proximal to such point of occlusion, the arterial vessel may become dilated and its endothelial lining hypertrophy until the lumen becomes practically atretic. The concentric corpuscles represent hypertrophied cells of the epithelial (entodermal) stroma. As these cells enlarge they press upon surrounding stromal cells and thus acquire a capsule of flattened cells. That these capsular cells are at least largely mesenchymal elements of the reticular stroma is demonstrated by the presence of reticulum fibrils.

The fundamental question concerns the nature of the factors underlying hypertrophy on the part of certain of the entodermal stromal elements. There is no conclusive evidence that it has normal functional significance. The evidence supports better the conclusion that the hypertrophy represents a pathologic process, perhaps a reaction to toxins accumulating as a result of the involution of the thymus, as suggested by Hammar ('21). There is no evidence of keratinization on the part of these corpuscles. None was seen with basophilic globules or granules, so characteristic of the multicellular corpuscles of rabbit and guinea-pig. Though it has not been demonstrated microchemically that these granules are keratohyalin, their resemblance to the

granules of the stratum granulosum of thick skin adds plausibility to Kingsbury's ('28) and Dearth's ('28) conception of the significance of Hassall's corpuscles. However, the histologic data concerning the thymus of the turtle give no support to this hypothesis.

As regards the interpretation of Jordan and Horsley ('27), who explain certain corpuscles of the mammalian thymus as areas of segmental atresia of capillaries and arterioles, the new data compel a modification. Such modification concerns only the primary cause of corpuscle formation and the more extensive involvement of epithelial stroma in the follicular types. The initial focus of corpuscle genesis is commonly extravascular, not intravascular as originally supposed. The first morphologic link in the chain of causation leading to multicellular concentric corpuscles of follicular type is a hypertrophy of epithelial stromal cells about a blood vessel. This produces a stenosis or atresia of the involved vessel. The central cells of such a corpuscle are endothelial cells and occasionally, erythroplastids and leucocytes. The enveloping cells may comprise both epithelial and mesenchymal stromal cells. Such origin explains both the central occurrence of endothelial and blood cells and the presence of true reticular fibrils. The 'keratohyalin' granules develop within the epithelial stromal cells. Proximal to such foci of arterial atresia, may develop corpuscles of the follicular type, described and illustrated by Jordan and Horsley ('27), some without appreciable inclusions of epithelial stroma. If the evidence from the turtle thymus may be legitimately applied to conditions in the mammalian thymus, certain follicular varieties of corpuscles may be assumed to arise also by secondary central liquefaction of original solid forms, which may become subsequently invaded by leucocytes.

The intimate genetic relationship between many of the concentric corpuscles and atretic blood vessels in the mammalian thymus may again be emphasized. There exists a fairly sharp reciprocal numerical proportion between medullary blood vessels and thymic corpuscles. Such is strikingly evi-

dent in a thymus of a nine-month infant which died of pneumonia. Here blood vessels are exceedingly abundant; corpuscles are very few in number and of small size. On the other hand, where corpuscles abound blood vessels occur only sparsely. Such relationship is shown in exceptionally extreme condition in sections of a thymus of the duck. Here occur very numerous corpuscles, many of large size and plasmodial form; blood vessels, especially of smaller size, are very few in number. If we should decide, as seems legitimate, that only concentric corpuscles with a constituent of epithelial (entodermal) stroma, however meager, are properly designated Hassall's corpuscles, then many mammalian thymic corpuscles are not genuine. However, in ordinary preparations true Hassall's corpuscles that lack basophilic content ('keratin') cannot be distinguished from simulacra which represent involution and atresia of blood vessels.

The regression of the thymus involves considerable tissue destruction in elements other than the thymic corpuscles. Such tissue destruction stimulates the appearance of granulocytes. These cells are exceedingly abundant, especially in the capsule and trabeculae of the turtle's thymus. They are accompanied by lymphocyte-like cells from which they apparently develop. These cells differ from typical lymphocytes mainly in the amount of cytoplasm. Many of these cells undergo regressive changes into typical plasma cells with vacuolated cytoplasm and into cells with Russell bodies. In developing into eosinophilic granulocytes, the 'plasmoidocytes' (Maximow, '27) begin as cells with only basophilic granules. Numerous transitional stages occur in which the granules include both basophilic and eosinophilic varieties. The evidence favors the conclusion that the lymphocytes of the thymus may under certain conditions function as hemoblast ancestors of eosinophilic granulocytes.

SUMMARY

1. Unicellular concentric corpuscles of Hassall constitute the predominating variety in the thymus of the box-turtle, *Terrapene (Cistudo) carolina*. These may occur in larger or smaller groups, but are more generally scattered singly, chiefly in the medulla, to some extent in the cortex. Multicellular and follicular varieties, with and without inclusions, also occur in varying numbers.

2. The unicellular thymic corpuscles represent hypertrophied epithelial (entodermal) stromal cells. The hypertrophy involves the accumulation of a hyaline acidophilic material in the cytoplasm coincident with degenerative changes in the nucleus. The hyalinized cytoplasm shows a series of concentric striations. Application of the Foot technique for reticulum and the Kopsch technique for Golgi apparatus and mitochondria gives results suggesting a more fluid, relatively acid condition of these striations. Anomalous nuclear division figures suggest an agonal effort at mitosis. There is no evidence of any attempt at keratinization.

3. As the epithelial stromal cells enlarge to become concentric corpuscles, they acquire a closely enveloping sheath of flattened cells resembling an endothelium. Application of the Foot technique reveals an extensive network of reticulum fibrils in connection with this sheath. The constituent cells are at least largely mesenchymal elements.

4. Fusions of smaller discrete epithelial stromal cells result in the formation of solid multicellular forms of thymic corpuscles. Liquefaction of central areas effects the production of follicular or cystic varieties. These may become secondarily invaded by parenchymal cells.

5. In the light of new data regarding the formation of Hassall's corpuscles in the turtle's thymus, the numerous corpuscles of the mammalian thymus in which blood vessels are involved must be interpreted in terms of the primary operation of extravascular rather than intravascular factors. The essential morphologic factor is the hypertrophy of epithelial stromal cells. This may secondarily produce stenosis

of adjacent blood vessels, with enlargement of endothelial cells and partial or total atresia of the vascular channel. The resulting corpuscles may consist of a central mass of hypertrophied endothelial cells and local aggregations of blood cells, and peripheral concentric layers of epithelial (entodermal) and reticular (mesodermal) cells.

6. The thymus of the box-turtle contains numerous lymphocytes, 'lymphoidocytes,' true plasma cells, basophilic and eosinophilic granulocytes, and cells with so-called fuchsophilic bodies of Russell. All of the varieties of cells trace their ancestry to the lymphocytes. The definitive eosinophils contain bacillary granules. These develop from eosinophils with spheroidal granules, which in turn develop from cells with basophilic granules. The abundant occurrence of granulocytes with mixed basophilic and eosinophilic granules supports the interpretation of granulocytes with orthobasophilic granules as unripe eosinophils.

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PLATES

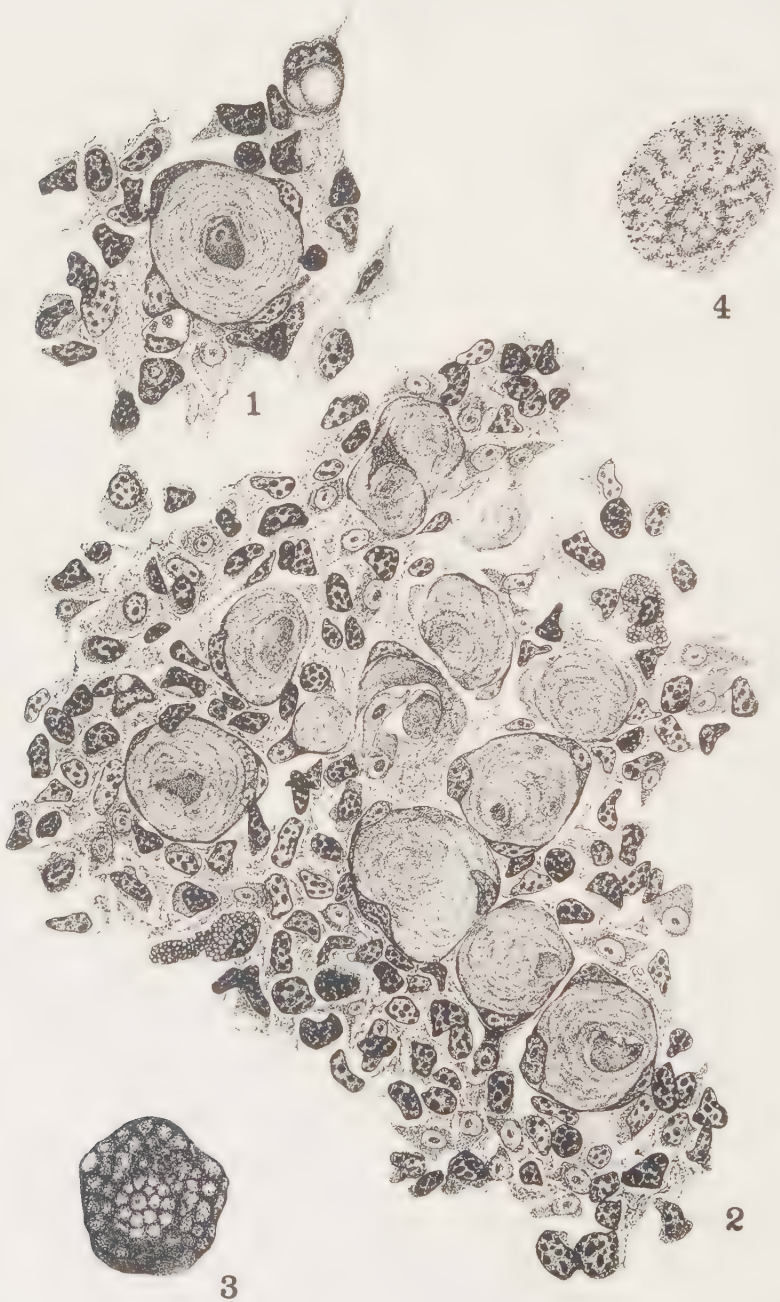


PLATE 1

EXPLANATION OF FIGURES

1 Typical unicellular thymic corpuscle of the box-turtle, closely enveloped by a membrane of flattened reticulum cells simulating endothelium. Of the outlying cells, those with the darker nuclei belong in general to the reticular stroma and lymphocytes, those with pale nuclei (below, at right) to the epithelial (entodermal) stroma. Helly fixation, Giemsa stain. Magnification, 1500 diameters.

2 Cluster of unicellular thymic corpuscles of various sizes and stages of development. The two corpuscles along the upper border are in process of fusion. The one in the center is fragmenting. The enveloping tissue consists of mingled reticular (mesodermal) and epithelial (entodermal) stroma, with lymphoid cells and a few eosinophilic granulocytes. Helly fixation, Giemsa stain.

3 Unicellular thymic corpuscle, stained according to the Foot technique for reticulum. The lighter central area indicates the position of the nucleus.

4 Similar corpuscle, stained according to the Kopsch technique for mitochondria and Golgi apparatus.

Drawings by Margaret Hasse Looper.

PLATE 2

EXPLANATION OF FIGURES

5 Unicellular thymic corpuscle, like that of figure 3, drawn under stronger illumination.

6 Multicellular thymic corpuscle, in process of formation by fusion of four epithelial cells. The surrounding cells are mostly lymphoid elements.

7 Multicellular thymic corpuscle, in process of acquisition of a lumen through dissolution of several central cells.

8 Multicellular (polynuclear, syncytial) thymic corpuscle with lumen. The lumen contains a disintegrating hyaline mass, remnant of central constituent entodermal cells. Below, at the right, appears a typical unicellular corpuscle.



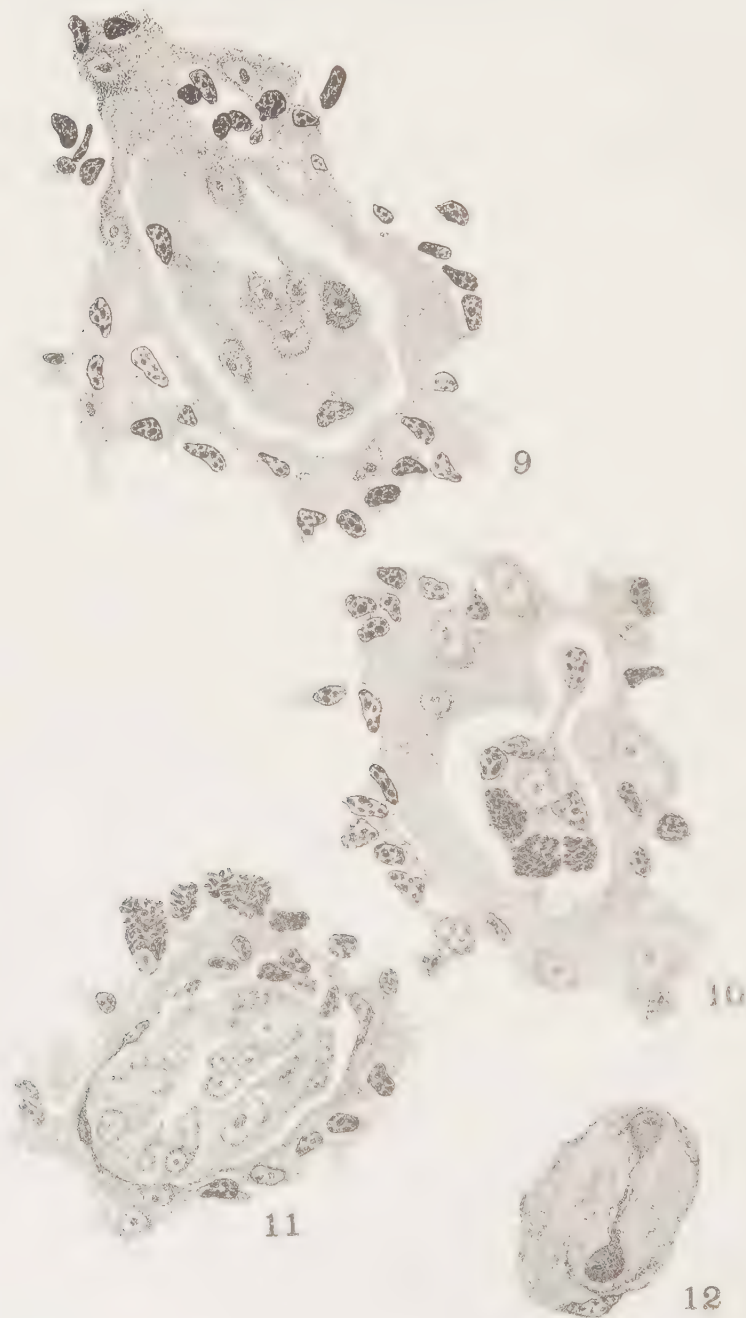


PLATE 3

EXPLANATION OF FIGURES

9 Similar multinucleated (plasmodial) thymic corpuscle, with central disintegrating mass separating from the periphery. Helly fixation, Giemsa stain. Magnification, 1500 diameters.

10 Similar corpuscle, invaded by three lymphocytes and three eosinophilic granulocytes which have fused with a central epithelial cell.

11 Transverse section of an arteriole from the same slide as figures 9 and 10. The endothelium has become greatly hypertrophied, almost obliterating the vascular lumen.

12 Monocellular Hassall corpuscle, in which the nucleus is dividing by amitosis. The process simulates mitosis more or less closely. The end result is a binucleated monocellular corpuscle, resembling the bicellular product of fusion.

PLATE 4

EXPLANATION OF FIGURES

13 Small area of box-turtle's thymus, stained to show the distribution of the reticulum. Except for the capsule (above) and a trabecula (at right), the reticulum is practically confined to the blood vessels. Eosinophilic granulocytes are especially numerous in the trabecula. Helly fixation, Foot staining technique. Magnification, 1500 diameters.

14 Preparation similar to figure 13. The area contains two unicellular corpuscles, each closely enveloped by a reticular sheath practically identical with endothelium.

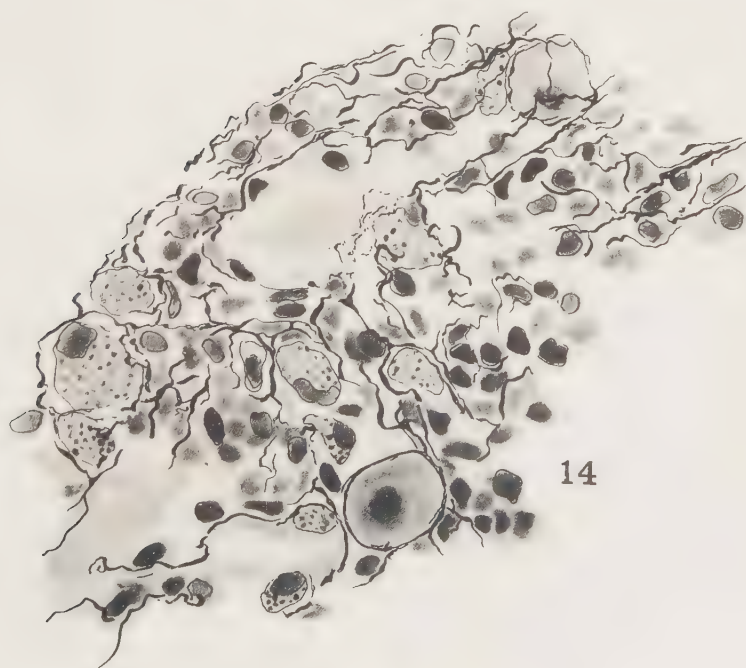
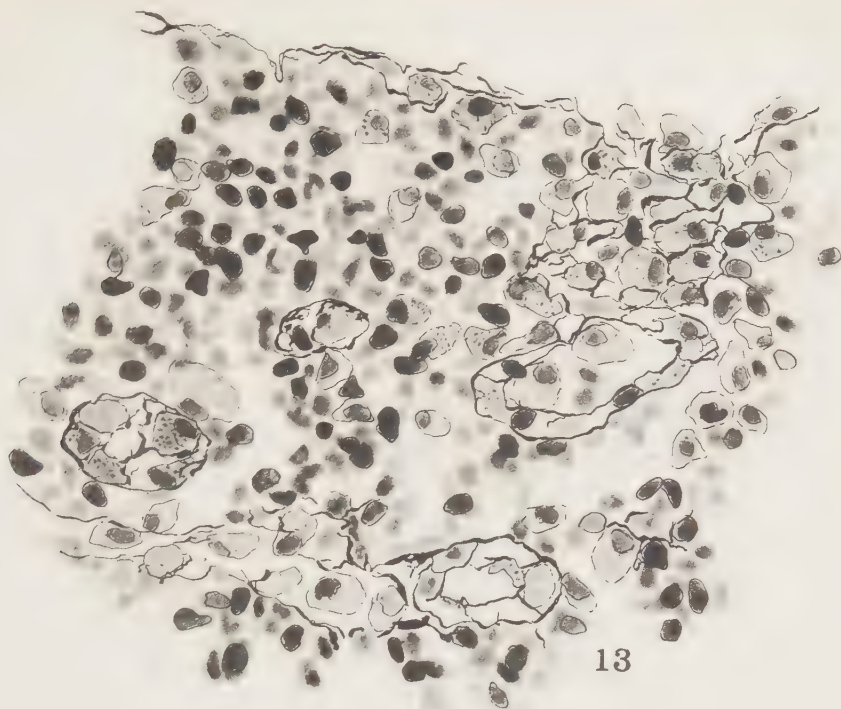


PLATE 5

EXPLANATION OF FIGURES

15 and 16 Lymphoid cells, ancestors of basophils (fig. 17), eosinophils (figs. 18 to 22), plasma cells (figs. 23 to 25), and Russell-body cells (fig. 26). Helly fixation, Giemsa stain. Magnification, 1500 diameters.

17 Basophilic granulocyte, an immature eosinophil.

18 Granulocyte with mixed basophilic and eosinophilic granules.

19 Mature eosinophilic granulocyte with spheroidal granules.

20 Eosinophilic granulocyte, in process of transition from spheroidal to ellipsoidal granules.

21 Definitive eosinophilic granulocyte with ellipsoidal and bacillary granules.

22 Group of six granulocytes, including four 'hybrids' at successive stages of ripening of the granules and two terminal eosinophilic granulocytes.

23 to 26 Successive stages in the transformation of a lymphoid cell into plasma cells and a terminal cell with a large hyaline 'Russell-body.'

27 Trinucleated cell, probably a plasma cell, with eosinophilic crystalloid bodies.

28 and 29 Two typical unicellular thymic (Hassall) corpuscles, with enveloping reticulum. Note laminated condition of the cytoplasm. Helly fixation, Giemsa stain. Magnification, 1500 diameters.

30 Unicellular thymic corpuscle. The central nucleated portion has separated from the peripheral exoplasm, giving the appearance of a hypertrophied endothelial cell lying within the lumen of a blood vessel.



A CASE OF POTENTIAL FREEMARTINS IN CATS

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SIX FIGURES

In a preliminary paper the author discussed the genetic significance of fused placentae in cats ('28 c). The findings of Hughes and Hoadley for swine, of Lillie, Keller, Bissonnette, and others for cattle, and of Bonnevie for cats, were also discussed as bearing on the wider application of the theory of the freemartin of Lillie and Keller and Tandler. It was shown that Doncaster ('20) and Little ('20) were within the bounds of possibility when they suggested that placental vascular anastomosis between male and female cat embryos might be the cause of the sterility of male tortoiseshell cats, since the case here described is evidence that such anastomosis does sometimes occur and at a stage in development preceding visible sexual differentiation, just as in cattle. Lack of such a case as the one described in the present article caused Bamber ('22) to seek to account for the sterile tortoiseshell tomcats on a genetic basis.

MATERIAL AND METHODS

This pair of twin kittens, members of a litter of seven, was found in a cat dissected by the class in comparative vertebrate anatomy in Trinity College. The cat had been killed the previous summer with illuminating gas, injected through the aortic arch with a carmine-starch-formalin injection mass for arterial dissection, and then skinned before being immersed in 10 per cent formalin for storage. At the time of injection, the abdomen had been partly opened to admit the preservative.

Subsequently, on exploring the abdomen farther and exposing the whole uterus, it was observed that there were six swellings in it, three on each side, with well-marked constrictions between them. One of these swellings next to the left ovary was larger than the others and was chosen for opening on this account. The uterine wall was slit lengthwise and a cross cut made at each end of the swelling, and the section of uterus removed and gently spread flat, leaving the zonary placenta complete and undamaged. The ends of the barrel-shaped placenta were thin-walled and transparent, while the sides formed the usual thick and vascular placenta proper. This was then slit open lengthwise from one thin-walled end to the other. It was found to be well injected with the mass originally introduced into the maternal circulation. The uterine wall had split so as to leave a covering of mucosa with the foetal part of the placenta.

On spreading apart the sides of the placenta, two embryos (14 and 14.5 mm. in crown-rump length) were found in place of the single embryo which had been expected. They were not in a common chorion, but in two distinct chorions, separated from each other by a thin-walled partition like the membranous chorion at the ends of the placenta, and each had its own proper amnion and rather large triangular yolk sac. The umbilical cords were attached to the interior of the placenta at some distance from each other. Blood vessels could be seen plainly, extending out from the distal ends of the umbilical cords and over the inside of the placenta (fig. 2).

On careful inspection of the exterior of the placenta, a slight groove was found to extend around it at about the equator, midway between the thin-walled ends. This was at the same level as the thin-walled partition on the inside, separating the two chorio-allantoic cavities. These findings and the separate yolk sacs and amnions of the twins, as well as the distance between the points of attachment of the umbilical cords to the placenta, made it clear that this was a case of fused placentae in cats, so complete as to make the zone of union almost indistinguishable (fig. 1). Inspection of the cut

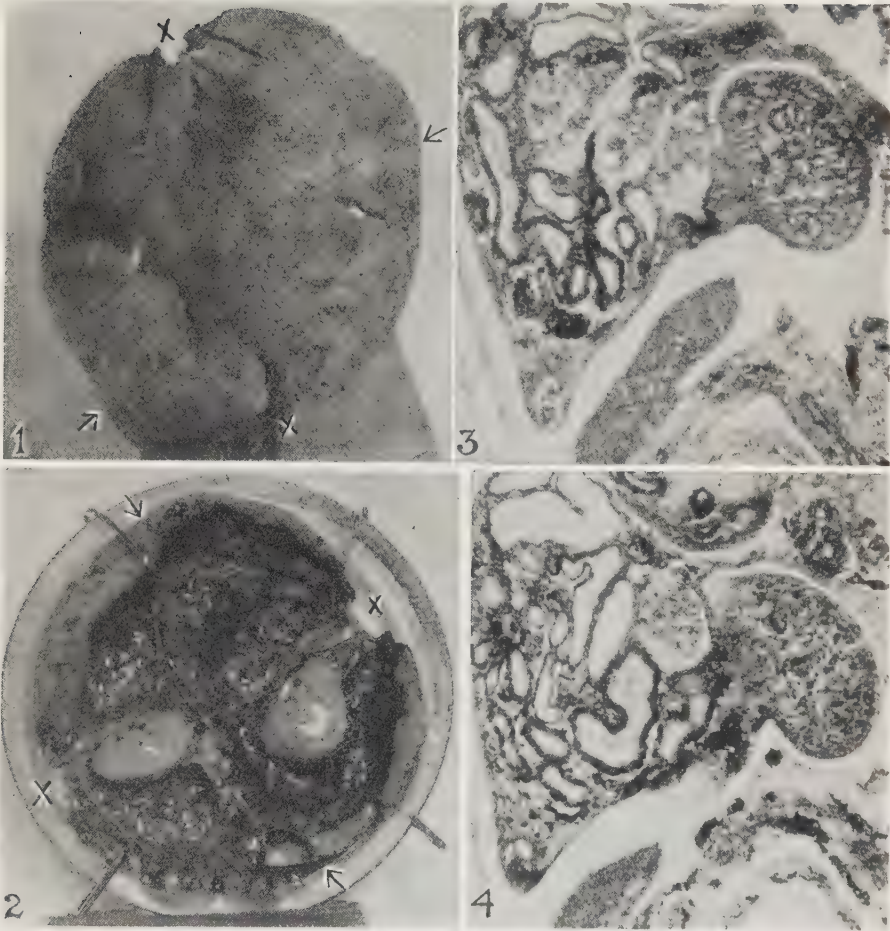


Fig. 1 External view of fused cat placentae. $\times 61/60$. X, X, thin parts of chorions at ends of placentae. Arrows mark line of union of the two original placentae.

Fig. 2 Internal view of fused cat placentae. $\times 61/60$. X, X, thin parts of chorions at ends of placentae. Arrows mark ends of line of union of the two original placentae.

Fig. 3 Transverse section through gonads and mesonephros of twin embryo kitten no. 1, showing an indifferent stage of the gonads, and relations of wolffian and müllerian ducts at this level. $\times 77$ approx.

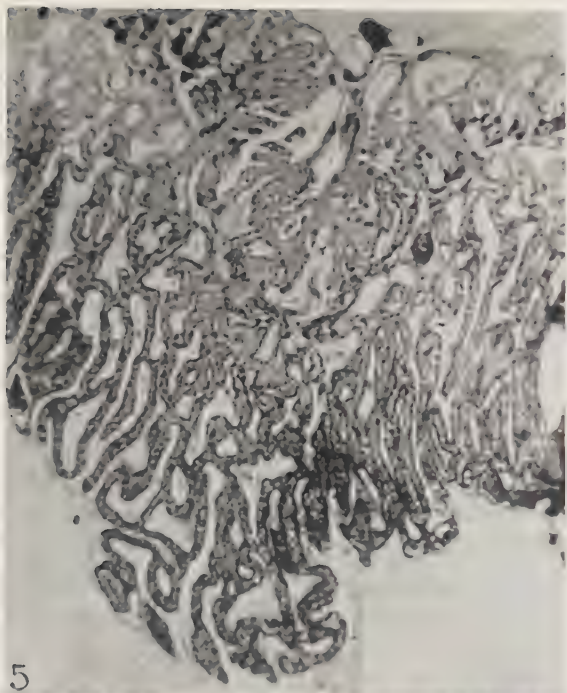
Fig. 4 Transverse section through gonads and mesonephros of twin embryo kitten no. 2, showing an indifferent stage of the gonads, and relations of wolffian and müllerian ducts at this level. $\times 77$ approx.

edges of the placenta where the cut crossed the groove at the equator revealed a slight ridge on the inside of the combined placentae opposite the groove on the outside, and affording attachment to the membranous partition.

A small piece about 6×4 mm. in dimensions was taken from the region of union, including part of it, and was embedded in paraffin and sectioned. The series of sections so obtained was stained in iron-hematoxylin on the slide and mounted in balsam. Photomicrographs were made of characteristic sections from within this piece (figs. 5 and 6). As seen in the photographs, the union is so complete that no separations are evident between the circulations of the two placentae, either on the maternal side, where the injection mass is found in blood vessels and lacunae, or on the foetal side. Both foetal and maternal blood appear to circulate freely on both sides across the zone of union.

The intimate anatomy of this type of placenta is well described and illustrated in Jenkinson ('25, p. 231, fig. 147 c) or in Shumway ('27, p. 114, fig. 73 c). In the present case separation must have occurred through the region showing dilatations of glands in the uterine mucosa below the glandular detritus and compacta layers, which here evidently constitute the decidua. In one of the sections photographed it might be supposed that there was discontinuity of the villi or lamellae of the twins on the foetal side of the placenta (fig. 6); but the other sections, all within 4 mm. of this one, show no such apparent break. These sections show plainly the groove on the outer or maternal side of the placenta (upper side in the photographs) and the corresponding ridge on the inner or foetal side, to which the blood vessels of the embryos and the chorionic membranous partition of the pla-

Figs. 5 and 6 Sections across line of union of two original placentae at different points (with internal foetal surface of placenta lowermost). These show the distinct inner or chorionic and allantoic portions, and the outer or uterine portions of the vascular placentae, the groove in external surface, and the vascularized partition between the separate placental cavities. Both inner and outer regions of the placentae are continuous and show no evidence of discrete vascular systems for each embryo. $\times 31$.



Figures 5 and 6

centae are attached. This inner ridge is particularly well shown in figure 6 and the outer groove in figures 5 and 6. In all cases there is a clear line of demarcation between the lamellae of the foetal part and the glandular region of the maternal part of the placenta or uterine mucosa.

In figures 1 and 2 the thin-walled ends of the membranes are shown at X, and the arrows mark the ends of the groove in the outside of the fused placentae and the ridge on the inside. The strands extending across the placenta in figure 2, from the region of the arrows, and passing between the embryos, are remnants of the membranous partition between the chorionic or allantoic cavities. Blood vessels may be seen on the inside of each placenta extending from the umbilical cords. The dark patches on the outside (fig. 1) are due to the injection mass from the maternal arterial system. As seen in figure 2, the heads of the embryos adjoin the region of union. Their relative sizes can also be made out, the right one in the figure being about half a millimeter longer in crown-rump length than the other.

Figures 3 and 4 are parts of sections through the bodies of the twin embryos at the levels of the gonads and show them to be in the so-called sexually indifferent stage, in which no difference can be discerned between testis and ovary. In both sets of gonads the primary sex cords are evident, but no signs of either tunica albuginea or secondary sex cords have yet appeared.

DISCUSSION

It is evident from the foregoing description that this is just the situation postulated by Doncaster ('20) and Little ('20) as a possible explanation for the sterile tortoise-shell tomcats, and it is proof conclusive that such a fusion is not only possible in cats, but that it may occur at a stage in embryonic development prior to the sex differentiation of testes and ovaries. Bamber ('22) was unable to find any cases as completely fused as these are; but she found no reason to doubt the possibility of such complete union. She

did, however, report some cases of coherent placentae in cats. The present case is also interesting because of its possible relation to the sterile female tortoise-shell cat described by Bonnevie ('25) and attributed by her to genetic or chromosomal aberration.

Hartman ('20), in his report of cases which he took to be the reverse of the freemartin in the opossum and in man, mentions a case of fused placentae in dog embryos which was communicated to him by Prof. H. M. Evans, of California. So far as the writer can discover, this one in cats is the only case of placental fusions with possible vascular anastomosis and hormone transfer from embryo to embryo in utero among carnivores, since accurate and detailed description and figures are lacking for the dog twins. Hartman also mentions some other cases of this sort reported long ago as occurring in dogs and men. These cases were not well authenticated and accurate and detailed descriptions of the situations were not given.

Recently, Hughes and Hoadley report such cases, with well-defined modifications of the freemartin type, for swine. Hoadley's ('28) was a case of 32-mm. length and Hughes ('27) described a series of three cases of such sex intergrades, 4.5, 6, and 9 cm. long, respectively, with details of histological structure of the reproductive organs and the arrangement of the foetal membranes. These studies leave no doubt as to the wider application of the theory of the freemartin to swine as well as to cattle.

While the sex-organ situation of the two cat embryos here described does not give any proof that the transfer from male to female twin of male hormone is capable of producing partial sex inversion in cats, as in cattle and swine, it does prove that the placental mechanism for such transfer sometimes occurs, even at stages preceding those at which in cattle the hormone is believed to be first produced in embryonic testes. So the possibility of cat freemartins depends on the susceptibility of the sex equipment of the female to the male hormone and on the production of

such hormone by the male gland at a time when some parts of the female sex organs are still dependent—that is, differentiating and susceptible to it. That such a hormone is present at some stages in cat development is hardly open to doubt; for cats exhibit much the same sort of sexual dimorphism as do cattle and swine, and castration in cats is followed by much the same effects as in dogs and cattle and other mammals. The question as to the degree of susceptibility of the female embryonic soma to the action of such hormone is not touched by this case. Should the cat react to such hormone in the same manner and degree as cattle, we may expect the transformation of the embryonic ovary into an infantile and more or less cryptorchid type of testis and, in rare cases, a descent of these testes into and even through the inguinal canal to the position equivalent to that of the ‘high flanker’ testis in males, just at the place of attachment of the scrotal sacs. This situation, even without sufficient enlargement of the clitoris to be equivalent to an infantile penis, would correspond very well to the sterile male as found among tortoise-shell cats. Should this be accompanied by modifications of external genitalia such as are well described and figured by Keller ('22) for cattle freemartins, it would be well-nigh impossible for even the expert in sex development in cats to distinguish between such a freemartin cat and a sterile male caused in any other way. Should the cat be susceptible to more marked modifications than cattle, both externally and internally, as a result of failure of cloacal and external genital derivatives to attain the self-differentiating condition before the onset of hormone activity, almost if not quite complete sex inversion might result in cats. This would account not only for the occasional sterile tortoise-shell tomcats, but also for the even more rare fertile ones. Bissonnette ('26) showed that descent into a scrotum appears to be necessary for complete spermatogenesis in male and freemartin testes in cattle and therefore for complete functional sex inversion.

Crowding in the uterus in early stages of pregnancy and embryonic development appears to be the cause of this situation in cats as well as in cattle and swine.

SUMMARY

1. The placental relations of twin cat embryos, 14 and 14.5 mm. crown-rump length, are described with their microscopic sexual anatomy.

2. Sections through the region of union of the fused placentae are described and figured and show no barrier between the placental circulations of the twins in a piece about 6×4 mm. in area.

3. This is a case of very complete fusion of vascular placentae necessary to condition the transfer of male hormone in utero from embryo to embryo and make possible the freemartin type of intersexuality among cats, as was suggested by Doncaster and Little to account for the occasional occurrence of male tortoise-shell cats and their almost uniform sterility.

4. It makes possible among cats the freemartin types of sex intergrades which have been studied so long among cattle and recently shown to be found under similar circumstances among swine. This widens the field among animal groups in which hormonal sex inversion may be expected.

5. The fusion necessary to furnish the mechanism for such hormonal modification is shown to occur in cats prior to the visible histological differentiation of testis and ovary.

6. It is also suggested that, with the small degree of difference between male and female external genitalia in cats, a freemartin cat with descended testes, even without modification of external genitalia, might easily be mistaken for a sterile male; and that, if the external genitalia and cloacal urogenital derivatives in cats have remained dependent—continuing to differentiate till after the onset of hormone action—and are dependent on it for sexual differentiation, almost if not quite complete sex inversion by hormone action may take place in cats.

7. In cattle there are freemartins with modifications of external genitalia in the male direction which, if they occurred in cats, would make such freemartin cat almost indistinguishable from a sterile male, no matter how produced.

8. Should such external transformation include the development of a scrotum with complete descent of the transformed gonads into it, it may be possible for complete spermatogenesis to occur in the freemartin cat gonad. This would account for the rare cases of fertile male tortoise-shell cats as the result of completely functional sex-inverted freemartin cats.

9. Early crowding in the uterus is suggested as the probable cause of this condition in cats as well as in cattle and swine.

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A NOTE ON MITOCHONDRIA

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ONE HELIOTYPE PLATE (TWENTY-TWO FIGURES)

INTRODUCTION

In the course of a series of experiments which I have been making on the influence of environment on cell structure, I have had occasion to fix tissues in fluids both with and without acetic acid, and in several cases have been surprised to find in the former, structures apparently identical with those which I have identified in the latter as mitochondria. This led me to make a careful study of these bodies in tissues taken from several different animals and preserved in various fixatives, some of which did, while others did not contain this reagent.

It has been generally assumed that acetic acid in a fixative destroys the mitochondria, but I do not know of any adequate critical tests to determine this. Fauré-Fremiet ('10, p. 502) says of the mitochondria in Protozoa: "Elles résistent aux acides et aux bases; elles sont insolubles dans tous les solvants des graisses." He also employed Zenker's fluid and Flemming's strong solution for fixing mitochondria, both of which solutions contain acetic acid in 5 per cent strength. Cowdry and Olitsky ('22), in a comparative study of mitochondria and bacteria, found that the former were 'occasionally and imperfectly preserved' in Zenker's fluid containing acetic acid and in sublimate-acetic.¹ They consider that "This persistence after fixation in Zenker's fluid with acetic acid and in

¹ Their tables (pp. 527 to 530), however, show only one case in which a trace of mitochondria persisted after treatment with the latter reagent.

sublimate-acetic is unusual and may be due to the rapidity of dehydration" (i.e., p. 531). These results apparently contradict the generally accepted opinion.

The mere fact that acetic acid used alone will dissolve mitochondria does not prove that it will do so when combined with other reagents. It is well known that various fatty substances (including mitochondria) are rendered relatively insoluble in fat solvents, such as alcohol, ether, etc., by previous oxidation in chromic compounds, so it is not improbable that, in such fixatives as Zenker, the bichromate may, in some cases at least, oxidize the mitochondria before the acetic acid (in 5 per cent solution) has time to dissolve them.

Treatment of tissues with unmixed acetic acid (even in 5 per cent solution) will, in most cases, dissolve the mitochondria; but it is mostly those near the surface of the tissue which are attacked, the deeper-lying ones being less affected.

I have also tested the solubility of mitochondria in alcohol of 80 per cent or 85 per cent strength and in ether, primarily with a view to determining the nature of the 'rods of Heidenhain' in the kidney, the mitochondrial nature of which has been questioned by some investigators. Thus, Policard ('10) questions their identity, basing his doubt upon the fact that the 'rods' do not break up or become granular, as is frequently the case with mitochondria.

"Ces différences sont très importantes à notre avis et suffisent pour montrer que ces formations ne sont pas de même nature. Mais la s'arrêtent les renseignements fournis par la morphologie" (i.e., p. 227). He admits, however, that Benda's suggestion that the 'rods' are absolutely identical with the mitochondria, which have been adapted to a certain function, is very plausible and is not contradicted by anything.

As we shall see later, the 'rods' frequently do break up into granules, in similar fashion to mitochondria elsewhere.

Cowdry ('18, p. 122) admits the validity of Policard's question, but in section VI of "General cytology" ('24, p. 316) he figures the kidney cells of a white mouse containing the characteristic 'rods,' which he describes in the accompanying legend as mitochondria.

The question, then, may be fairly said to be open.

Regarding the solubility of mitochondria in fat solvents, such as alcohol and ether, Fauré-Fremiet (l.c.) also differs from most authors, for in several places in addition to that cited above, he speaks of their insolubility in these reagents.

In my own work I have found that alcohol and ether affect the mitochondria differently in different experiments. In many cases they are present, though much broken, due, probably, to the poor fixation, while in others they appear to be more or less completely dissolved.

METHODS

In the table at the end of this paper I have given the kinds of tissues used and the animals from which they were taken, the treatment employed and the results. In every case the pieces of tissue were small, not over 3 to 4 mm. in diameter (or thickness), and usually much less than this. The time of fixation varied with the method employed. Zenker's fluid² was allowed to act from one to several hours; Champy 'overnight,' while with Regaud I followed the usual procedure as outlined in Lee ('24). In the latter, however, acetic acid, when used, was added only to the first bath, being allowed to act for about twenty-four hours. Regaud fixation was always done in an ice-box.

For staining I have used Heidenhain's iron haematoxylin and acid fuchsin, the latter counterstained with methyl green. With the latter method I usually find that the fuchsin stains too strongly, while the methyl green is mostly extracted in dehydration. Accordingly, I have usually brought the fuchsin to the steaming-point (requiring about thirty seconds), then washed the slide in water, stained in methyl green for one to two minutes, run the slide through 95 and 100 per cent alcohols saturated with methyl green, into clove oil, and then into xylol. The method of course has to be adapted to each individual tissue, and no exact procedure can be given.

² My Zenker fluids have all lacked the sodium sulphate.

The drawings have all been made with the camera lucida, but in most cases the mitochondria have been filled in by hand. Wherever possible, however, they have been outlined with the camera, as indicated in the description. The optical combination employed was a Bausch & Lomb no. 10 hyperplane ocular and a 1.8-mm. fluorite oil-immersion objective, giving a magnification of 1400 diameters.

OBSERVATIONS

In the table referred to above I have summarized the results of my experiments. Some details of these results will be given here. It is hardly necessary to state that not all of my preparations were successful whether made with or without acetic acid. With the exception, however, of the chicken, to which reference will be made in the proper place, I succeeded about as often with the acetic fixatives as with those lacking this reagent.

1. *Frog (Rana pipiens)*

Sections of the liver of this species were undoubtedly taken from different animals, although my records do not state whether they were or not, for they present two very different pictures. In one (figs. 1 to 4) the cells are larger, very much vacuolated, with the mitochondria grouped about the nuclei and wall, while in the other (fig. 5) the cells are smaller, are not vacuolated, and the mitochondria are uniformly distributed throughout.

I have found this vacuolated condition occasionally in the liver of other animals and am at a loss to interpret it. It undoubtedly is a shrinkage phenomenon, but the condition of the protoplasm must be different in the two types of cell, since the same fixatives produce different effects in each. In Zenker preparations, both with (fig. 1) and without (fig. 2) acetic acid, the mitochondria appear as granules or short rods, similar in abundance and distribution in both. Comparing the sections as a whole, there is no evidence of any effect of the acetic acid on the mitochondria.

The same (vacuolated) type of liver cell is shown in figure 3 from a specimen treated for about one hour in 85 per cent alcohol and then fixed in Zenker without acetic acid. The mitochondria appear as granules (occasionally as short rods) arranged in strings or rows. There is no evidence here of a reduction of the mitochondria, although their distribution in adjoining cells is somewhat irregular. I have not, however, attempted any exact quantitative determination, relying on general appearance of the tissue as a criterion of their abundance.

A somewhat similar appearance is presented in figure 4, which shows the vacuolated type of cell after treatment with ether for about an hour. The mitochondria here are small granules, with a few short rods. They are irregular in distribution, being abundant in some, few in other cells.

Figure 5 shows the other type of frog liver. Here the mitochondria, in the form of numerous irregular granules, are uniformly distributed through the cell, save for some slight shrinkage about the periphery. A specimen of this type of liver, which had been treated for about one hour with 85 per cent alcohol and then fixed in Zenker without acetic, showed no evidence of any effect of the alcohol on the abundance of the mitochondria.

Treatment of frog's liver with 5 per cent acetic acid for an hour removes all but a few faint traces of the mitochondria from the peripheral cells (fig. 6), while in the deeper-lying cells, where the acid has not penetrated, they are still intact (fig. 7). I believe this experiment proves that the structures I have designated mitochondria really are such, and not some artefact.

The granules in the kidney cells, which are generally considered to be mitochondria, but whose identity has been called in question, are well preserved both in Zenker with and without acetic acid. Tissue fixed in the former is shown in figure 8, while that fixed in the latter is shown in figure 9. Save for minor differences, both pictures are similar, there being no evidence of any effect of the acid on the mitochondria.

In this case also tests with 5 per cent acetic by itself indicate the mitochondrial nature of these structures, for they are almost, if not entirely, dissolved in tissues so treated.

Treatment of the kidney with ether and with 85 per cent alcohol yields unsatisfactory results. The fixation is very poor, so that it is impossible to be certain whether the mitochondria have been dissolved or not.

2. Fish

Results similar to those in the frog have been obtained with the black spotted trout (*Salmo clarki*), and the minnow (*Pimephales promelas*).

Both in sections of the liver of the trout fixed in Zenker minus acetic and in those fixed in Zenker plus acetic the mitochondria are clearly evident, being somewhat more numerous in the second preparation than in the first. In the latter the nuclei may stain differently in adjacent cells. Most of them take the methyl green, but occasionally they stain with fuchsin. In many other of my preparations also the nuclei take the fuchsin stain instead of the green. There is apparently nothing specific about these stains, their action depending on the time of staining and the degree of extraction.

Figures 10 and 11 show kidney tubules of the trout, the former from a Zenker minus acetic preparation and the latter from one fixed in Zenker plus acetic. In both the 'rods' show well, there being no evidence of any effect of the acetic acid when acting with the chrome salt.

A preparation of the spleen of this species fixed in Zenker plus acetic shows numerous granular mitochondria, while preparations of the intestine give similar results whether fixed in Champy with or without acetic acid. In these latter the mitochondria appear as fine granules scattered through the cells, but mainly massed at the surface (fig. 12).

Preparations of the liver of the minnow, fixed in Champy with and without acetic acid, give pictures which are similar to each other in every respect, one of which is shown in

figure 13. The mitochondria, which are relatively few in this tissue, occur as granules, rods, or small masses, scattered irregularly through the cells.

3. *Chicken*

With this tissue (both adult and embryonic) I have found mitochondria oftener in material fixed in Zenker, Regaud, and Champy without acetic acid than in that fixed in similar fluids containing acetic. In a few cases, however, I have obtained good results in acetic material, which are shown in figures 14 and 15. In figure 14, taken from the limb of an embryo of a few days' incubation and fixed in Zenker plus acetic, the mitochondria, most of which were outlined by camera, occur as rods or strings of granules, many of considerable length—perhaps twice the diameter of the nuclei. Figure 15 is from a tissue smear from an embryo of several days' incubation. The mitochondria show clearly as numerous rods and granules scattered through the cell. In several other smear preparations fixed in acetic-acid mixtures I have obtained good demonstrations of mitochondria, which usually appear as (small) spherical or ovoid masses, rather than as rods, while in others I have been unable to do so.

4. *Mammals*

Among the mammals I have used the white rat (*Mus rattus*) and the guinea-pig (*Cavia cobaya*) for my experiments.

In sections of the liver of the rat fixed in Zenker plus acetic (fig. 16) the mitochondria, many of which were outlined by camera, appear as irregular bodies, varying in size from small granules to masses about the size of a red corpuscle. In the slide from which this figure was made the nuclei stain red as well as the mitochondria. In yet other slides the latter, as well as the nuclei, stain green or purple, depending on degree of extraction of the stains, as previously noted.

Pieces of liver fixed in Zenker both with and without acetic acid show practically the same mitochondrial content. There is, however, a difference in different preparations in the

amount of coalescence of the mitochondria. This tendency appears to have no relation to the presence or absence of acetic acid, but does appear to be increased by 50 per cent alcohol or ether (fig. 17).

Coalescence of mitochondria has been previously noted by many authors, especially in the formation of the 'nebenkern' in spermatogenesis and the yolk nucleus in oogenesis.

Fixation of rat's kidney in Zenker either with or without acetic acid has no apparent effect on the mitochondria, which appear as numerous granules, aggregated mainly in the basal two-thirds of the cell (fig. 18).

In rat kidney treated with ether there is some evidence of solution of the mitochondria. While they are distinct in some tubes, they are scarcely discernible in others. Many of them appear here, in the form of rings, suggesting a possible solvent action of the ether on a central, more soluble portion, leaving a more resistant shell unaffected (fig. 19). There is some evidence here, which, taken together with experiments on guinea-pig tissue, to be described below, indicates that the mitochondria are composed of two substances, one more, and the other less soluble in a fat solvent, such as ether.

Guinea-pig liver was fixed in Zenker's fluid, both with and without acetic acid. It was also treated with 5 per cent acetic acid for ninety to one hundred minutes, followed by fixation in Zenker minus acetic. Both Zenker fluids give similar results (figs. 20, 21). The protoplasm is much vacuolated in many of the cells, reminding one of the conditions seen in one of the frog livers described above. It is less so near the surface of the liver and the large blood vessels. The mitochondria are coarse, granular, or rod-like bodies equally well preserved in both Zenker fixatives. In fresh liver smears the mitochondria appear as numerous minute refractive granules, some of which stain sharply in Janus green. In smears from pieces of material treated for ninety minutes with 5 per cent acetic acid they are absent or scarce. They do not blacken in osmic-acid fumes, but come out sharply in smears fixed in Champy's fluid.

In Zenker material the mitochondria, and even the nuclei in some cases, stain readily with fuchsin, whereas in material which is first treated with 5 per cent acetic acid for ninety to one hundred minutes and then fixed in Zenker without acetic, followed by eighteen hours' mordanting in 3 per cent potassium bichromate in the piece, and subsequently for eleven to twelve hours on the slide, the fuchsin stain has very little effect. With the former material the fuchsin was allowed to act only long enough to bring it to steam (about thirty seconds), while the latter was stained for five or six minutes in fuchsin and the methyl green was extracted as far as possible in alcohol followed by clove oil. Even after this treatment, designed to strengthen the fuchsin stain so far as possible, it shows but little effect. In many of the cells, however, occur granules similar to those which I have identified as mitochondria in Zenker material, but they are largely or entirely dissolved in others. Figure 22 represents the conditions fairly well. The granules are irregular, mostly in the form of rods, strings, or circles, occasionally dots. The evidence here indicates that the mitochondria are composed of two substances, one readily soluble in acetic acid (a phospholipin) and the other an albumin which is relatively insoluble, as suggested by previous authors (Cowdry, '18, p. 83).³

In the few slides of guinea-pig kidney which I have made the results are not very satisfactory, as the preparations are rather poor, but neither in material fixed in Zenker with acetic acid nor in that treated with 80 per cent alcohol for ninety to one hundred minutes and followed by Zenker without acetic is there any evident diminution in the mitochondria, as compared with material fixed directly in Zenker minus acetic acid.

³ See also page 358 above.

CONCLUSIONS

The experiments which form the basis of this paper indicate that:

1. Acetic acid in the strength (5 per cent) usually employed in fixatives is not necessarily injurious to mitochondria, when used in combination with a chrome salt, as in Zenker's, Champy's, or Regaud's fluids. Taken by itself, however, it will usually dissolve them more or less completely.

2. Treatment of tissue with ether or strong alcohol does not necessarily destroy mitochondria, although in some cases it apparently does so. The preservation of the tissues after treatment with these reagents is usually so poor that their effect on the mitochondria cannot be definitely determined.

3. The 'rods of Heidenhain' in the kidney tubules resemble mitochondria in every respect, both in form and in the readiness with which they break up into granules; in staining reaction, solubility in dilute acetic acid, and in their resistance to this reagent when combined with chrome salts. In my experiments on the frog kidney, however, they appeared to be more soluble in alcohol than were the mitochondria in the liver; but this last result is uncertain, as already explained.

4. Mitochondria sometimes agglomerate in larger masses, and this tendency may be increased by treatment with alcohol.

5. Treatment with acetic acid and with ether, notably in guinea-pig tissue, supports the view that the mitochondria are composed of two substances, one a protein and the other a phospholipin.

TABULATION OF EXPERIMENTAL RESULTS

SPECIES	TISSUE	FIXATIVE	RESULTS
Rana pipiens	Liver	Zenker + acetic	Positive
Rana pipiens	Liver	Zenker - acetic	Positive
Rana pipiens	Liver	85 % alcohol	Positive
Rana pipiens	Liver	Ether	Positive
Rana pipiens	Liver	5 % acetic	Negative
Rana pipiens	Kidney	Zenker + acetic	Positive
Rana pipiens	Kidney	Zenker - acetic	Positive
Rana pipiens	Kidney	85 % alcohol	Uncertain (positive in some experiments; apparently negative in others)
Rana pipiens	Kidney	Ether	Uncertain (positive in some experiments; apparently negative in others)
Rana pipiens	Kidney	5 % acetic	Negative
Salmo clarki	Kidney	Zenker - acetic	Positive
Salmo clarki	Kidney	Zenker + acetic	Positive
Salmo clarki	Spleen	Zenker + acetic	Positive
Salmo clarki	Liver	Zenker + acetic	Positive
Salmo clarki	Liver	Zenker - acetic	Positive
Salmo clarki	Intestine	Champy - acetic	Positive
Salmo clarki	Intestine	Champy + acetic	Positive
Pimephales promelas	Liver	Champy + acetic	Positive
Pimephales promelas	Liver	Champy - acetic	Positive
Gallus domesticus	Connective tissue (embryo)	Zenker + acetic	Positive
Gallus domesticus	? (embryo)	Zenker + acetic	Positive
Gallus domesticus	Several	Zenker, Champy, Regaud, both with and without acetic	Few positive; many negative; former mostly in fixatives without acetic
Mus rattus	Liver	Zenker - acetic	Positive
Mus rattus	Liver	Zenker + acetic	Positive
Mus rattus	Kidney	Zenker + acetic	Positive
Mus rattus	Kidney	Zenker - acetic	Positive
Mus rattus	Kidney	Ether	Uncertain, positive in some tubules; apparently negative in others
Cavia cobaya	Liver	Zenker + acetic	Positive
Cavia cobaya	Liver	Zenker - acetic	Positive
Cavia cobaya	Kidney	{ Zenker + acetic Zenker - acetic 80 % alcohol }	Uncertain, mitochondria apparently present, but broken
Cavia cobaya	Liver	5 % acetic	Partial solution of mitochondria

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DESCRIPTION OF FIGURES

Figures 1 to 22 are camera drawings, $\times 1400$. The stain is fuchsin and methyl green, except as otherwise noted.

PLATE 1

EXPLANATION OF FIGURES

- 1 Frog liver, fixed in Zenker with acetic.
- 2 The same, fixed in Zenker without acetic.
- 3 The same, treated with 85 per cent alcohol, followed by Zenker without acetic.
- 4 The same, treated with ether, followed by Zenker without acetic.
- 5 The same as figure 2, from a different frog.
- 6 The same, treated with 5 per cent acetic and stained in iron haematoxylin, followed by Zenker without acetic. Section taken near liver surface.
- 7 The same material as in no. 6, but section taken farther from surface.
- 8 Frog kidney, fixed in Zenker plus acetic.
- 9 The same, fixed in Zenker without acetic.
- 10 Trout kidney, fixed in Zenker without acetic.
- 11 The same, fixed in Zenker with acetic.
- 12 Trout intestine, fixed in Champy with acetic.
- 13 Minnow liver, fixed in Champy with acetic acid and stained with iron haematoxylin.
- 14 Chick, embryonic connective tissue fixed in Zenker with acetic. Mitochondria mostly outlined by camera.
- 15 Isolated cell from a smear of embryo chick, fixed in Zenker with acetic and stained in iron haematoxylin.
- 16 Rat liver, fixed in Zenker with acetic. Some of the mitochondrial masses were outlined by camera.
- 17 The same, treated with 50 per cent alcohol followed by Zenker without acetic and stained in iron haematoxylin. The larger mitochondrial masses were outlined by camera.
- 18 Rat kidney, fixed in Zenker with acetic.
- 19 The same, treated with ether, followed by Zenker without acetic.
- 20 Guinea-pig liver, fixed in Zenker without acetic.
- 21 The same, fixed in Zenker with acetic. Most of the mitochondria were outlined by camera.
- 22 The same, treated with 5 per cent acetic, followed by Zenker without acetic.



ADDITIONAL OBSERVATIONS ON INTERNAL MIGRATION OF THE OVUM IN THE SOW AND IN THE GUINEA-PIG¹

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ONE TEXT FIGURE

INTRODUCTION

Although it has been nearly seventy years since Kussmaul first pointed out that there are two possible pathways for migration of the ovum, it is only recently that interest in the problem has been revived, chiefly through the work of Corner on the sow in 1915 and 1921. Although the evidence supplied by the latter author seemed conclusive enough as regards internal migration of the ovum in the sow, the present writer determined to test the question experimentally. Since he began this work his attention was called to a paper published by Warwick in 1926 dealing with intra-uterine migration of the ovum in the sow and reporting the same type of experiment. Nevertheless, he feels that this additional evidence should be reported, together with a discussion of the work of some investigators whose papers seem to have escaped notice.

In the guinea-pig, under similar conditions (that is, with one ovary removed), it has been shown that internal migration of the ovum does not occur. Since, no doubt, the reason for this lies in the structure of the uterus, an examination of this organ in the guinea-pig has seemed pertinent.

¹Contribution no. 1, series B, from the Department of Anatomy, Medical Department of the University of Georgia. This work has been aided by a grant from the National Research Council through its Committee for Research in Problems of Sex.

REVIEW OF THE LITERATURE

Corner's paper for 1921 depicts diagrammatically the two possible pathways for migration of the ovum as stated by Kussmaul: the first taking place across the abdominal cavity; the second, from one uterine tube to the other by way of the body of the uterus (or, in forms with bicornuate uteri, from one horn to the other). As far back as 1876, Mayrhofer(8) considered that migration of the human ovum occurred as often as once in ten ovulations. For a discussion of these human cases (such as reported by Williams, Kelly, Winckler, and Andrews) the reader is referred to Corner's paper. One of the most recent cases of what apparently was external migration of the human ovum was reported by Slivinsky(12).

All writers on this subject refer to the work of Leopold in 1880. This investigator removed the ovary on one side (in the rabbit) and the uterine horn on the other, and then bred the animal. He demonstrated pregnancy in the remaining uterine horn by this method, and apparently proved external migration of the ovum for this form.

Kinney, in 1924, showed that internal migration of the ovum did not occur in the guinea-pig. He reported on nineteen animals from which one ovary had been removed.

It is very interesting to note that quite different results have been obtained in the rabbit, as regards external migration of the ovum, both by Baur, in 1921 and 1922, and by Parkes, in 1924. Baur reports five occurrences of pregnancy in twelve rabbits after removal of the ovary on one side and after resection of the tube on the other side (with transplantation of the stump intramuscularly). He concludes that external migration of the ovum occurs in the rabbit and that it is not seldom or accidental, but of a compensatory nature. In the following year, he demonstrated that internal migration of the ovum does not take place in the rabbit. He then performed laparotomies on three pregnant animals and found the following situation:

Rabbit no. 1. Right horn, 5 young; left horn, 3 young. Right ovary, 2 corpora lutea; left ovary, 6 corpora lutea.

Rabbit no. 2. Right horn, 6 young; left horn, 4 young. Right ovary, 8 corpora lutea; left ovary, 2 corpora lutea.

Rabbit no. 3. Right horn, 3 young; left horn, 3 young. Right ovary, 2 corpora lutea; left ovary, 4 corpora lutea.

He concludes: "this division of the ova can happen only by external migration." If Baur's observations in these three animals were correct, it is hard to see why he did not get twelve pregnancies instead of five in the experiment referred to above. Moreover, one would get the impression from these three instances that external migration in the rabbit was even more common than internal migration in the sow.

In contradistinction to the results reported by Baur, Parkes found that, after ovariectomy on one side and salpingectomy on the other in six rabbits, not a single conception occurred, though the does were mated successfully for from three to four months. He even thinks that Leopold's results may have been due to regeneration of the excised ovary, though he admits that external migration may occasionally occur. Apparently, he did not know of Baur's work, as he made no reference to it.

Warwick, whose paper has been referred to above, definitely proved internal migration of the ovum in the sow. He removed one ovary from each of four gilts and, after breeding them, found young in both uterine horns in three of them; one did not conceive.

EXPERIMENTS ON THE SOW

Out of six gilts that were semispayed on August 17, 1927—the operation consisting of the removal of a large portion of one uterine tube, together with the ovary of that side²—two were bred during the first week in February, 1928, shortly after reaching sexual maturity.

After approximately two and a half months, both animals were sacrificed. In the one in which an ovariectomy had

² The writer desires to acknowledge his indebtedness to Dr. T. G. Brooks, of the Department of Surgery, for aid in the operations.

been done on the left side, three males and one female fetus were found in the right horn of the uterus and one female fetus in the left horn. The right ovary contained eight well-developed corpora lutea.

In the second case (the one from which the right ovary had been removed) there were six fetuses on the left side (three males and three females) and four on the right side (three males and one female). The left ovary contained thirteen corpora lutea.

These cases therefore substantiate the claim that internal migration of the ovum occurs in the sow.

EXPERIMENTS ON THE GUINEA-PIG

The uteri of six freshly killed guinea-pigs were grossly examined in order to discover the nature of the uterine body, which, when viewed from without, seems to be single in structure. In addition, the bodies of some of these specimens were sectioned by hand, either beginning at the upper end or at the cervical end. In either case it was immediately evident that the uterine body in this species is bipartite, that each half is a distinct tube without communication with the other side until the internal os of the cervix is reached.

In length it is about 1 cm. long and in general outline conforms rather closely to that of the sow. However, while there is no external indication of a separation of the uterine body into two separate halves, my observations lead me to conclude this is the case. The partition extends all the way to the single cervix, thus making internal migration in this form practically out of the question. This will explain why Kinney could report four fetuses in one uterine horn with no migration to the opposite side, where there was none, and shows that a comparative study of the uteri of various forms with reference to the problem of internal migration is in order.

From a level just above the internal os a block of tissue was removed, fixed in Bouin's fluid, sectioned on the lower (cervical) end, and stained with haematoxylin and eosin.

Figure 1 is a microphotograph made of one of the sections. It shows that the condition in the guinea-pig uterus is not essentially different from that in animals that have a bipartite cervix (with two canals) as well as a bipartite uterine body. As it is hardly to be expected that an ovum could pass so far down and then up on the other side, this deep subdivision of the uterus is sufficient to account for the



Fig. 1 Cross-section of the body of a guinea-pig uterus just above the internal os of the cervix. Note the layer of longitudinal muscle surrounding the whole and the two distinct layers of circular muscle, one around each uterine canal. $\times 34$.

absence of internal migration in the guinea-pig. Nor is external migration indicated in this species, since none of the four fetuses reported by Kinney migrated to the other horn of the uterus.

DISCUSSION

From these experiments it is quite clear that a further knowledge of the comparative anatomy of the ovary, bursa ovarica, and of the uterus is needed in order to determine the

possible course that an ovum may take after ovulation. Whether external or internal migration of the ovum (if either) occurs in a given species seems to depend upon a number of factors, the two most important being the degree to which the ovum is covered by the bursa ovarica and the morphology of the lower uterine segment in animals with bicornuate uteri. The importance of the latter has already been discussed. The significance of the former is well illustrated by the rat and mouse (as recorded by Robinson (11)), in which animals the bursa separates the ovary from the peritoneal cavity so completely that external migration in these forms is impossible. Nor can internal migration occur, since the rat has two cervical canals. Curiously enough, Zuckerkandl(14) includes no description of the rat and mouse in his extensive paper on the comparative anatomy of the ovarian bursa; nor, it seems in reviewing Robinson's paper, did he see the latter's description of the bursa in these forms, although it was purely for this purpose that Robinson wrote his paper.

At the opposite extreme from the rat and mouse is the condition of the human ovary, in which the peritoneal bursa is almost wide open. However, even in the human considerable sacculation of the ovary may occur, as shown by Reynolds(10). In the guinea-pig the slit in the bursa is large enough to permit the extrusion of the ovary, but it is doubtful that an ovum would pass from it into the peritoneal cavity. Even strong suction from ciliary movement or peristaltic action of the opposite tube would doubtless influence it little.

Finally, we come to the question of the significance of internal (as well as external) migration. Is it a device for proper spacing in the uterus, in the interest of better nutrition (as Corner has suggested) or is it merely a fortuitous matter, the eggs migrating wherever there is opportunity? In either event there seems to be a great difference in the ability of animals to profit by this adjustment. In such types as the human, both internal and external migration of

the ovum seem to be possible (although the latter is more frequent); in other species only one method is available (external migration in the rabbit); whereas, in a third group (rat and mouse) the morphological arrangements are such as to prevent any form of heterolateral migration.

CONCLUSIONS

1. Internal migration of the ovum in the sow is substantiated by the experimental method of unilateral ovariectomy.

2. Internal migration of the ovum probably occurs in all forms in which there is free access from one uterine cornu to the other through a uterine body, provided that sufficient ova are fertilized to make such spacing desirable.

3. In some forms in which internal migration is impossible, on account of the anatomical configuration of the uterus, external migration may occur, as in the rabbit, though this is probably rare.

4. External migration is anatomically possible only in those forms that have an incomplete bursa ovarica.

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VAGINAL CURETTAGE AS A MEANS OF DIAGNOSING PREGNANCY IN THE GUINEA-PIG¹

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ONE TEXT FIGURE AND ONE HELIOTYPE PLATE (FOUR FIGURES)

INTRODUCTION

The fact that the vaginal epithelium of the guinea-pig, like that of several other rodents, undergoes a very marked transformation during pregnancy has been known for many years. Lataste(4) seems to have been the first investigator to make a note of this phenomenon ('87), and under his direction Morau(5, 6) published two papers bearing directly on the subject ('88, '89). Following these writers, articles appeared by Salvioli(8) and de Retterer(7), in 1890 and 1892. Since then this subject has been treated extensively in the literature.

Recently, the present writer(1) has taken up the investigation and followed it from a number of angles, working out the vaginal epithelial changes in the guinea-pig under a wide variety of conditions. This paper cites most of the literature in this particular field up to the present.

As stated in this article, the changes that occur in the vaginal epithelium are quite marked. The stratified squamous epithelium of estrus is transformed gradually to a high columnar, mucous epithelium (figs. 2 and 3). The writer has shown (see reference 1) that this change begins about

¹ Contribution no. 3, series B, from the Department of Anatomy, Medical Department of the University of Georgia. This work has been aided by a grant from the National Research Council through its Committee for Research on Problems of Sex.

the second week of pregnancy and is well developed by the end of the third week. From this time on the changes that occur are due in large part to the hypertrophy of the vagina, though the mucous nature of the cells becomes enhanced as pregnancy continues.

Between estrous periods the vaginal mucosa assumes a transitional form; it is very thin and cannot be described as strictly stratified squamous or simple columnar. For illustrations of this form see reference 1. Since no mucous cells are present in this epithelium, curettage at this interval will give negative scrapings.

METHODS

The extent of this epithelial transformation first came to my attention while working on the mechanism of the opening and closing of the vaginal orifice in the guinea-pig(2), a female at term having been sacrificed during this investigation. It occurred to me then that, if these cells could be scraped from the vagina, they would serve as a means of diagnosing pregnancy in this animal. Some time later, I happened to be examining vaginal smears from guinea-pigs daily, and in many of them I noticed the large mucous cells that I have since found are present in large numbers after abortions or deliveries (figs. 4 and 5). Their presence in such smears is due to their desquamation after the termination of the pregnant state. The appearance of the cells in these two figures is the same as when they are obtained by the method described below, that is, by vaginal curettage.

These cells are characteristic in appearance, being very large and reticulated, and having a small, dark nucleus. They are as a whole basophilic and take a pale blue or lavender stain. Usually they are seen in clumps, as shown in the illustrations; very seldom singly. By sectioning vaginas of animals that had been pregnant from seven to nine weeks, it was easy to identify these cells as the high columnar, mucous epithelial cells of pregnancy (compare figs. 3 and 5).

Since it has been found that very few of these mucous cells desquamate before delivery, the vaginal smear as first described by Stockard and Papanicolaou(9) is not satisfactory for obtaining cells from the vaginal wall as a diagnostic method. It is true, however, that many smears from pregnant animals will show quite a number of these cells; but all smears do not. Consequently, curettage seemed to be the best avenue of approach.



Fig. 1 The curette.

Some difficulty was experienced in devising a curette that would work satisfactorily. The epithelium was so smooth that the edge of the curette did not take hold easily. Finally, the type illustrated (fig. 1) was found to accomplish the purpose. It was made from a narrow, flat piece of steel by bending one end back to less than a right angle with the shaft and then sharpening the bent-over end to a fine edge.

The curettage is performed as follows: With the aid of an assistant, the animal is wrapped in a towel with only its

head out, in order to prevent struggling, and given just sufficient ether to get it asleep. The vulvar site is cleaned with a sponge saturated with alcohol and the vulvar lips torn open. The curette is now introduced and the curettage performed, pressing the instrument firmly against the surface scraped (usually the ventral wall of the vagina). With the end of a clean pair of forceps the curetted material is pushed from the angle of the curette to a glass slide and smeared there. Fixation is with alcohol-ether, equal parts; staining, with haematin and eosin. The entire procedure up to fixation requires but a few minutes, and the animal is usually awake by the time the slide is placed in the alcohol-ether.

In giving guinea-pigs ether it is advisable to use an indirect method, that is, to pump it from a bottle with a rubber bulb. The gas is thus delivered to the animal's nostrils through a rubber tube, on the end of which the half of an old rubber bulb with two or three air holes in its sides can be attached as a nose-piece. If ether is applied directly, post-operative pneumonia is apt to be frequent. Incidentally, we have never had any accidents whenever these methods have been used.

OBSERVATIONS

At times it is very desirable to know whether a female guinea-pig is pregnant or not. The long gestation period (nine weeks) tends to make this knowledge even more desirable if non-pregnant animals are needed within a short time for experimental work.

On January 18, 1928, we received a shipment of ten female guinea-pigs. These animals had been ordered for a particular experiment that required non-gravid animals. On January 23rd, vaginal curettage was undertaken, with the results shown in table 1.

As will be noted, two of these females had abortions the day following arrival—a not uncommon accident after long transportation to new surroundings. Of course, the scrapings from these animals were positive, though they were not then pregnant. A third animal was in stage 1 of estrus

the day after arrival. Naturally, it had a negative scraping three days later; but in examining the slides no identifying marks revealed from which animal they came, each diagnosis being made on the presence or absence of the characteristic cells.

Since the vaginal epithelium of the high columnar, mucous type does not develop until nearly three weeks after copulation, it can readily be seen that two curettages, three weeks apart, are necessary in order to rule out pregnancy. A curet-

TABLE 1

This shows the results obtained in curetting a new shipment of ten animals

NO.	FIRST CURETTAGE	SECOND CURETTAGE	FINAL DIAGNOSIS	CORRECT
1051	1/23/28, negative	2/24/28, positive	Positive	Yes
1052	1/23/28, doubtful	2/14/28, positive	Positive	Yes
1053 ¹				
1054	1/23/28, positive	None	Positive	Yes
1055	1/23/28, positive	None	Positive	Yes
1056 ²	1/23/28, negative	None	Negative	Yes
1057	1/23/28, negative	2/14/28, positive	Positive	Yes
1058	1/23/28, positive	None	Positive	Yes
1059 ¹				
1060	1/23/28, negative	None	Negative	Yes

¹ Aborted 1/20/28.

² Stage 1 of estrus 1/20/28.

Animal 1060 returned to estrus before time for the second curettage.

tage once a week for three weeks would be better. This explains why three of the animals referred to were first diagnosed as non-pregnant, though a subsequent examination revealed positive findings. They had been pregnant less than three weeks when they arrived.

It is worthy of note that, with this precaution, the final diagnoses were all correct (omitting the two animals that were known just to have aborted). In doing this work, especial care was taken to avoid bias. No palpations were made and none of the animals were big with young when they came. Finally, the slides were stained and mounted by an assistant,

and the examiner did not know one slide from another, but based his diagnosis only on the histological appearance.

In the spring of 1928, we were doing some experiments on artificial insemination of the guinea-pig by way of the ovarian bursa(3) and were very desirous of knowing as soon as possible whether a particular animal was impregnated or not. Of course, those that returned to estrus at the expected time were not pregnant, but about the others we could not be absolutely sure, since these animals may occasionally have estrus so weakly expressed that the vaginal orifice does not open. So in each of this group a curettage was done three weeks after the operation. Seven animals were operated upon. Three came back into estrus and the remaining four were curetted. All four of the latter showed positive scrapings and subsequently were found actually to be pregnant.

In trying out this method in the beginning a large number of curettages were done on animals whose state was known, but the scrapings were examined without knowledge of their source. It was soon found that it was not at all a difficult matter to tell whether a given slide was from a pregnant or non-pregnant animal.

DISCUSSION

In making such diagnoses three limitations must be borne in mind: First, the vaginal epithelium of immature females is of the high columnar, mucous type and practically identical with that of advanced pregnancy. Consequently, curettage on animals that have never come into estrus would show the mucous cells. The cells, however, are smaller than those of pregnancy, though the difference in size is not very great. These animals are very small and their sexual immaturity can usually be inferred from their size. While the majority of them have their first estrus at about three months of age, we have had quite a number that were only a little over two months old when the first stage-1 smear was obtained.

Secondly, curettage of animals within one or two weeks after abortion or delivery will probably reveal mucous cells

in the scrapings, since all the cells do not desquamate at the delivery estrus. Occasionally, the mucous cells may persist until the estrus following the delivery estrus, but not thereafter in a scraping will they be abundant, as from a pregnant animal, though in smears a few may be seen for two or three weeks longer. In a vaginal scraping from a pregnant animal the dominancy of the mucous cells is easily apparent.

A third limitation has already been mentioned, viz., the animal must be pregnant three weeks before the test is effective.

For those experienced enough to determine early pregnancy in the guinea-pig by palpation, this method may not hold much of value. It may provide a method of differential diagnosis in case of abdominal tumors, but this is doubtful. Still, many persons can not by palpation of this animal determine pregnancy until it is well advanced. For them vaginal curettage should solve the question. We have already found it practicable within its limitations and often very useful. Its theoretical value is not without interest.

Finally, the question arises as to the diagnostic value of this procedure in other species. We have not tried vaginal curettage in other animals, with one exception, and can say nothing about it. A student became interested in the method and tried it on white rats known to be pregnant, but without success. That is, he found no characteristic mucous cells. This work was not supervised and possibly the curettage was not properly done. It requires firm pressure to scrape the mucous cells off.

Of course, in animals with a persistent form of epithelium throughout pregnancy this method would have no value. For this reason it has no application in the human (with our present knowledge of the histology of the human vagina).

CONCLUSIONS

1. By vaginal curettage pregnancy can be diagnosed in guinea-pigs three weeks or more after copulation.

2. Positive scrapings can be obtained from sexually immature females and from females within two or three weeks after abortion or miscarriage; these are limitations of the method.

3. The method has no value, so far as can be told at present, in animals that do not have a marked transition in the vaginal epithelium during pregnancy, such as is seen in the guinea-pig.

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PLATE

PLATE 1

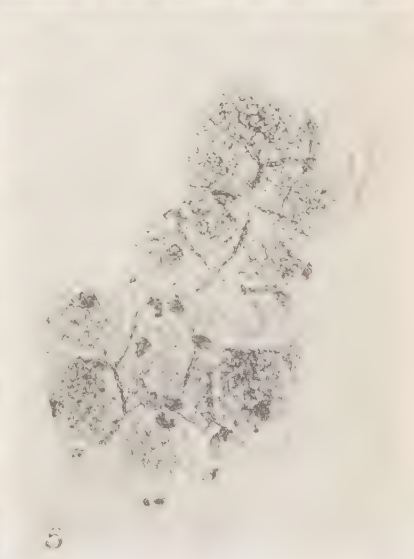
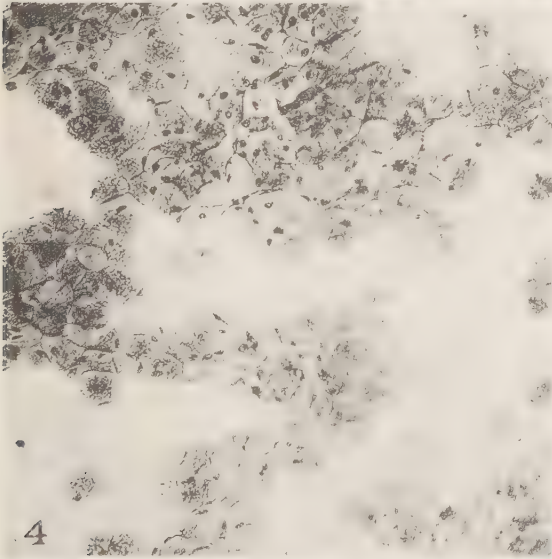
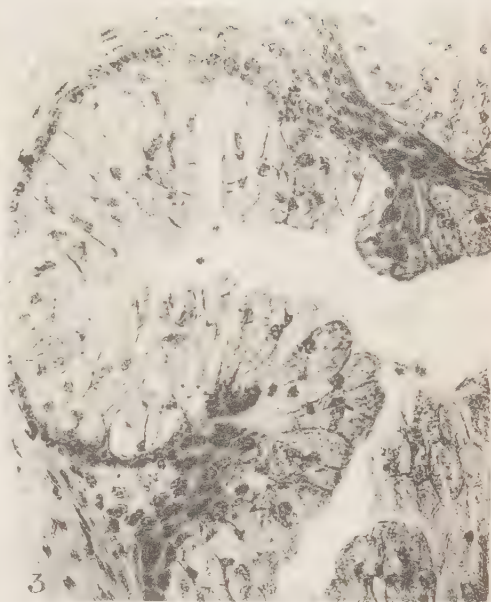
EXPLANATION OF FIGURES

2 Low-power microphotograph of vagina of a guinea-pig nine weeks pregnant, showing the high columnar mucous epithelium. $\times 150$.

3 High-power view of the same. Compare these cells with those in figure 5. Notice the difference in color and size of the nuclei in the two figures. $\times 360$.

4 Low-power microphotograph of mucous cells as they appear in a vaginal scraping. The collection in clusters is characteristic. $\times 150$.

5 High-power view of the same. Compare the size of the cells with that of the near-by leucocytes. Note that the nuclei appear to be undergoing caryolysis. The reticular structure of the cells is well shown here. $\times 360$.



FURTHER OBSERVATIONS UPON THE MINUTE STRUCTURE OF THE LABYRINTH IN THE PLACENTA OF THE SLOTHS

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ONE HELIOTYPE PLATE (ONE FIGURE)

In the *Comptes Rendus de l'Association des Anatomistes* (Liège, '26) there appeared an article by de Lange upon the structure of the placenta in *Bradypus*. According to the opinion of de Lange, the placenta of *Bradypus* represents an intermediate stage between the endotheliochorial and hemochorial types of Grosser. This conclusion is at variance with that arrived at by the present writer, who published a report simultaneously upon the placentation of the sloths ('27). From these observations the conclusion was drawn that the placental labyrinth of the sloths resembles most closely the structure of that found in carnivores and that the placenta is a transition form between the syndesmochorial type and the endotheliochorial type, but more closely approaching the latter.

The material studied by de Lange consisted of two specimens of *Bradypus*, one possessing an embryo measuring 47 mm. from snout to tip of tail; the other, 12 cm. from snout to tip of tail. The author's microscopic studies were made upon a series of twenty-five placentae of *Bradypus griseus griseus*, the youngest of which possessed an embryo measuring 47 mm. from snout to tip of tail; the most advanced, a fetus of 171 mm., near term.

In view of the conflicting conclusions arrived at in these two studies, the writer has deemed it advisable to reexamine

his material in the light of de Lange's description. The present communication gives the results and conclusions arrived at in the reinvestigation of the finer structure of the labyrinth.

The structure of the labyrinth may be divided for convenience into two stages, that observed in the youngest specimens and that encountered in the fully mature state.

The placental labyrinth consists essentially of lamellae of trophoblast, which inclose the maternal blood vessels, separated by bands of fetal mesenchyme. The question under consideration involves the exact nature of what constitutes the trophoblast and what forms the immediate walls of the maternal blood vessels or blood lacunae.

In the younger stages the lamellar structure of the labyrinth is not so pronounced, due to the only partial penetration of the trophoblast by the fetal mesenchyme. Hence, at the line of invasion of the trophospongia or intermediate zone of the placenta by the chorion, the trophoblast exhibits solid masses and tongue-like proliferations of chorionic syncytium. The trophoblast is everywhere syncytial, no cytotrophoblast being recognizable in even the youngest stages of the series. The plasmoditrophoblast is characteristic. It consists of broad bands or tongues of cytoplasm, completely syncytial, with well-defined limiting borders, slightly granular or flocculent cytoplasm, and nuclei heaped up in clumps at irregular intervals. The bands or tongues of plasmoditrophoblast inclose maternal blood vessels. The latter are invariably lined by a single layer of deeply staining cells which are interpreted, for reasons to be given, as modified endothelial cells belonging to the maternal vessels. It is these cells that de Lange rejects as being endothelial in origin and assumes to be a part of the plasmoditrophoblast.

The considerations which lead the present writer to interpret these cells as endothelial in origin are:

1. They are not arranged as a plasmodium, but possess cell boundaries. Hence, consideration of these as trophoblastic in origin would necessitate a reversal of our conception of

the relationship of cytotrophoblast to plasmoditrophoblast. The cytotrophoblast would be on the periphery or outside of the plasmoditrophoblast.

2. It can be observed in sections colored with Mallory's connective-tissue stain that the cells lining the maternal blood vessels rest in places upon a membrane which stains blue and is fibrous in character, and which intervenes definitely in some places between the layer of endothelial cells and the inclosing true plasmoditrophoblast. This membrane is interpreted as a remnant of the original wall of the maternal vessels. Moreover, occasionally a spindle-shaped cell is encountered in this membrane separating the endothelium from the trophoblast. These remnants of maternal tissue inclosing the endothelium bear witness that the labyrinth can be considered as intermediate between the syndesmochorial type and the endotheliochorial type.

3. The cells lining the maternal blood spaces are continuous with identical cells lining maternal vessels in the trophospongia (or intermediate zone); there is no doubt as to the endothelial character of these cells. Moreover, at the border of the invading multinucleate trophoblast one can observe the trophoblast encroaching upon, partially surrounding, or finally inclosing, endothelial-lined maternal vessels with no doubt as to the separate origin and identity of the two.

4. The cells lining the maternal vessels are modified endothelial cells and are not like ordinary capillary endothelium or that of veins. The endothelial cells in question bulge into the lumen of the blood vessels; they are relatively short and have round nuclei. However, there is good evidence in other placental material, in the trophospongia of rodents and more particularly in the labyrinths of the placentae of carnivores, that the endothelium can undergo such a change. In fact, the character of the endothelium is identical with that in the dog's or cat's placenta and the relationship of the endothelium to the surrounding trophoblast similar to, although not identical with, that found in the cat and dog. The writer agrees with Becher ('21) that the similarity between the

labyrinths in sloths and carnivores is very great, but with the reservation, which he fails to make, that there is still some evidence of a transition from the syndesmochorial type to the complete endotheliochorial type.

The layers separating the maternal from the fetal circulation in the labyrinth of the sloths are as follows:

1. The maternal endothelium, everywhere intact in the viable lobules.

2. A delicate fibrous reticulum, upon which the endothelium rests and which is probably incomplete in places. In this membrane an occasional spindle-shaped cell of maternal origin belonging to the vessel wall is met with.

3. The trophoblast, consisting in the youngest stages of broad strips or bands of multinucleated syncytial cytoplasm inclosing the maternal vessels.

4. The fetal mesoderm with the capillaries in close contact with the adjacent trophoblast; and in the stroma between the capillaries and also in contact with the trophoblast, large mesenchymal cells which later become epithelioid in character.

In the labyrinth of the mature placenta of the sloths one encounters the same layers as in the younger stages, except that they have been markedly modified, and without a knowledge of the earlier picture the interpretation would be difficult. Of course, in the lobules which undergo complete degeneration, as the placenta assumes in the gross its definitive form, the layers lose their relationships and intermingle in the degenerative process. However, in the viable lobules which have become the mature placenta, the layers are still recognizable. The endothelium lining the maternal blood vessels persists and keeps the same character as previously. It still retains the delicate incomplete membrane of reticular fibers upon which it rests. The layer of trophoblast undergoes a marked modification from the earlier condition. The trophoblast thins out tremendously to form finally a thin, scarcely recognizable membrane surrounding the maternal vessels, while at the same time the aggregations of nuclei in

the syncytium, characteristic of the earlier period, disappear almost totally, leaving only an occasional oval nucleus in their place. The capillaries in the fetal stroma, as before, lie in intimate contact with the trophoblastic membrane, and in the meanwhile the epithelioid cells derived from the stroma of the chorion have become almost epithelial in character, forming a sheet of cuboidal or polygonal cells interrupted only by the capillaries. These two structures (epithelial cells and capillaries) together press themselves against the thin trophoblastic membrane.

It will be recognized from this description that the finer structure of the sloth's labyrinth bears a close analogy to that of the carnivores. The author cannot agree with de Lange that the cells lining the maternal blood spaces are trophoblastic in origin and that they may be designated as pseudo-endothelial. Furthermore, it appears inadvisable to homologize the groups of deeply stained nuclei occurring in the trophoblastic syncytium in the young stages with the ectoplacenta of Duval.

In reference to the attachment to, and growth of, the placenta into the uterine mucosa, as well as in the general shapes which it assumes, the placenta of the sloth differs markedly from the known representatives of other mammalian orders. Certainly, there is little that is reminiscent of the rodents or of the other groups possessing so-called discoidal, hemochorial, labyrinthine placentae. Aside from the labyrinth, there is no great resemblance either in mode of growth or metamorphosis of the placenta to placentation as found in carnivores. Nevertheless, if a comparison had to be made, the analogies that could be drawn would be closer to carnivores than to the types of placentation encountered in groups other than the *Xenarthra* themselves. Thus, the rather diffuse and superficial penetration of the uterine mucosa by the trophoblast in the initial stages is more like the behavior of the trophoblast as seen in carnivores than in the groups with discoidal, hemochorial placentae. On the other hand, such striking features as the haematomata of the

carnivores and the tremendous development of uterine glands are strikingly absent in the sloths. It is merely suggested that the degenerative process that overtakes many of the lobules in the sloth may have a remote relationship phylogenetically to the haematomata found in carnivores and that, physiologically at least, the two processes may subserve the same functional needs. In a specimen of the placenta of one of the carnivores (*Cynogale*) at my disposal the formation of haematomata, beside occurring at the two poles of the small blastocysts, occurs in a widely diffused manner throughout the placenta in the form of small petechial patches, producing a marked superficial resemblance of the haematomata to the degenerative changes occurring in the sloths.

Nearly all of our knowledge thus far concerning placentation in sloths has been obtained from the study of the tri-dactyl form (*Bradypus*), whereas the didactyl form (*Choloepus*) has been studied only once (Turner, '75). Recently, the writer obtained a placenta and fetus of *Choloepus hoffmanni*, which it has been of interest to compare with the material from *Bradypus*. The fetus in this specimen measures 136 mm. length of spine without tail and possesses neither hair nor an epitrichial membrane. It is judged to be from the midgestational period. The placenta in the gross is essentially like the placenta of the three-toed sloth at the same period. It consists of irregularly distributed lobules of placental tissue covering much of the chorion, with membranous chorion in the gaps. Each lobule varies in size and thickness from 1 to 1.5 cm. in diameter and from 2 to 5 mm. in thickness. The lobules are predominately reddish in color in the fresh specimen, but in addition there is a smaller number of smaller nodules which are white or greenish in the fresh state. The latter are degenerating lobules and resemble similar ones observed in the placenta of *Bradypus*. Furthermore, there are thin brownish plaques in the chorion, the final state of degenerated lobules before their complete disappearance. The placenta is seen to be composed of diffusely

scattered nodules and differs from the placenta of *Bradypus* at this period of gestation in not showing a tendency of the chorion to become completely membranous at the cervical pole. Turner's specimen from the end of gestation is described as bell-shaped, occupying the fundic portion of the uterus. It is quite likely, from Turner's specimen and our knowledge of the metamorphosis of the placenta of *Bradypus*, that the placenta in the present instance would have eventually become localized in the fundic half of the uterus by further degeneration of lobules. The minute amniotic caruncles or villi which the author has described for the placenta of *Bradypus* are lacking in this specimen. The umbilical cord is smooth, as in *Bradypus*; its attachment and the distribution of the blood vessels supplying the placental lobules are identical with the conditions observed in the three-toed sloth.

Microscopically, the labyrinth of this placenta resembles very strikingly that of *Bradypus* in its mature form. The most conspicuous cell layer is the one composed of cells with prominent round nuclei which inclose the maternal blood vessels or blood spaces. This layer of cells rests upon a fibrous tunic which in many places appears to be in apposition to, or even in direct continuity with the fetal stroma between the maternal blood vessels. The fetal mesoderm is infiltrated by large epithelioid cells as in the tridactyl sloth. Moreover, as in the mature state of the labyrinth in *Bradypus*, the question arises as to what constitutes the trophoblast. Is it the conspicuous layer of cells inclosing the maternal blood spaces, as de Lange believes, or is it represented by the epithelioid cells, or, finally, is it constituted by some elements which on first examination escape notice because of profound reduction? The latter explanation appears to be the most reasonable one, judging from our experience with *Bradypus* and as the result of a careful study of the present specimen. For, in searching the labyrinth carefully, clusters of nuclei can be found, which differ from the nuclei of the vascular wall as well as from the epithelioid cells, and which intervene in places between the tunic surrounding the

maternal vessels and the fetal mesoderm. These cell clusters are seen in some regions to lie in a membrane of cytoplasm which extends for short distances between the vascular tunic and the fetal stroma. In many places, however, it has become beyond the power of the eye to detect this trophoblastic membrane and its presence might be totally denied, if it were not for the evidence at hand in *Bradypus* concerning the fate and ultimate marked reduction of the trophoblast. It is our belief, therefore, that the labyrinth of this single specimen of *Choloepus* should be interpreted as almost identical in morphology with the labyrinth of *Bradypus* of a comparable stage. The two points of possible difference that should be emphasized are the presence of a more pronounced vascular tunic around the maternal vessels than in *Bradypus*, and a more complete reduction of the plasmoditrophoblast which originally intervened completely between the maternal and fetal mesoderm.

SUMMARY

Study of a series of placentae of *Bradypus* and of one specimen of *Choloepus* has led to the conclusion that the placentae of these two forms show marked morphological resemblance in both gross and microscopic structure.

Moreover, examination of the placental labyrinths of sloths indicates that the labyrinths are transitional in character between the syndesmochorial and endotheliochorial types of Grosser. Among the existing placentae, the labyrinth exhibits the most pronounced resemblance to the type of labyrinth encountered in the Carnivora. The maternal vessels, of the caliber of small veins, retain their endothelium, which becomes, however, markedly modified. Furthermore, the vessels retain a thin tunic of fibrous tissue of maternal origin. The trophoblast incloses the maternal vessels in the early stages as a broad syncytial layer, which ultimately becomes markedly reduced to a thin sheet and possibly disappears entirely over large areas in the mature labyrinth. Within the fetal stroma at an early period epithelioid cells arise which arrange themselves along the border or periphery of the fetal mesoderm adjacent to the trophoblastic membrane.

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PLATE 1

EXPLANATION OF FIGURE

1 Drawing of a portion of the placental labyrinth of the tridactyl sloth (*Bradypus griseus griseus* Gray). The fetus (no. 24 of the author's series) to which this placenta belongs measures 94 mm. from snout to tip of tail. The area represented is typical of the placental labyrinth during the first half of the period of gestation. The cell layers constituting the labyrinth can be clearly seen. Inclosing the maternal blood sinuses (*m.b.s.*), modified endothelial cells (*en.c.*) are seen which are oval or polygonal in shape and have conspicuous oval nuclei. Beneath the endothelial cells occasional flattened cells (*sp.c.*) with spindle-shaped nuclei are visible. These constitute an interrupted layer of elements which represent remains of the original perivascular tunic surrounding the maternal vessels. The maternal vessels, composed of the endothelial cells and the accompanying spindle-shaped cells, are inclosed by broad bands of syncytial cytoplasm, the trophoblast or chorionic ectoderm (*syn.*). In this syncytial fetal structure clumps of nuclei can be observed. The chorionic ectoderm rests upon a delicate basement membrane which separates it from the cell elements of the fetal mesenchyme. In the fetal stroma (*f.m.*) capillaries (*cap.*) can be seen in close proximity to the chorionic membrane, as well as large mesenchymal cells with round or oval nuclei which are particularly abundant in the periphery of the fetal stroma.

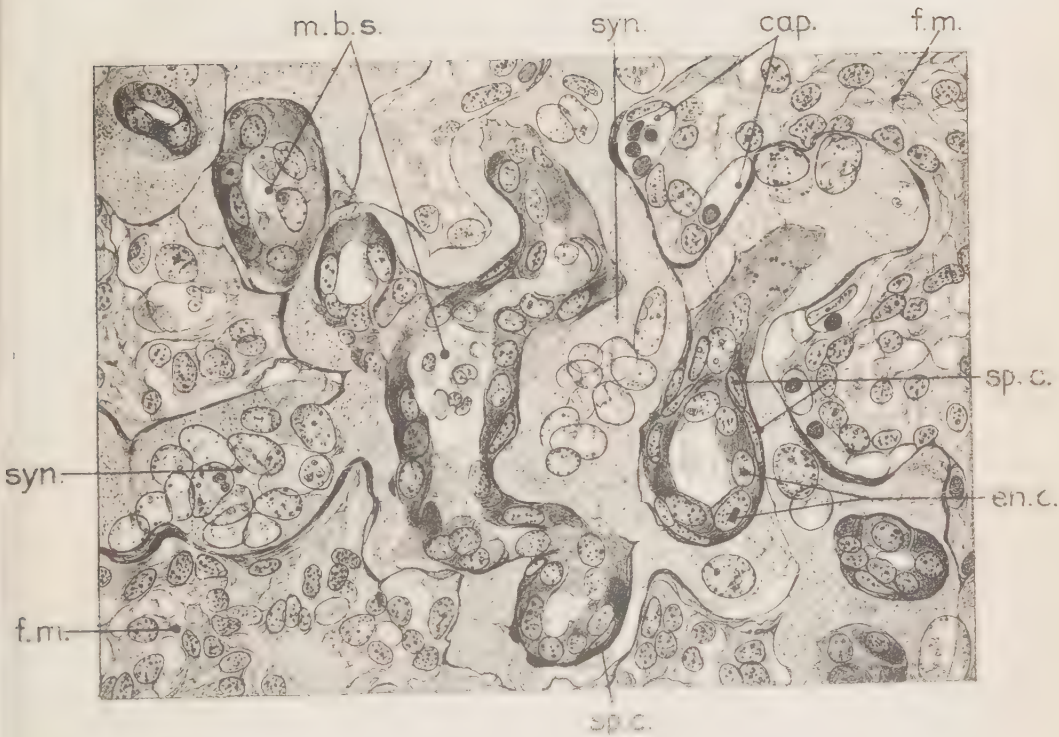
During the second half of gestation the picture of the labyrinth changes markedly. The syncytial chorionic ectoderm thins out to a scarcely perceptible membrane and loses its conspicuous clumps of nuclei which apparently undergo, for the most part, complete degeneration. At the same time the mesenchymal elements in the periphery of the fetal stroma gradually assume a more and more epithelioid character and arrange themselves about the capillaries as an almost continuous layer of cells at the zone of contact of the chorionic membrane with the fetal stroma. These two changes—the reduction of the chorionic ectoderm and the development of the epithelioid cells—produce an alteration in the appearance of the labyrinth during the later stages. Without a knowledge of the earlier stages, the interpretation of the definitive picture would be difficult.

The author wishes to express his appreciation to Mr. James F. Didusch for the excellent illustration.

ABBREVIATIONS

cap., fetal capillaries
en.c., maternal endothelial cells
f.m., fetal mesenchyme

m.b.s., maternal blood sinuses
sp.c., spindle-shaped cells
syn., chorionic syncytium (trophoblast)



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